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A METHOD OF BIOASSAY BASED ON THE DURATION OF ACTION OF ESTROGENS

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THE increasing therapeutic use of long-acting sex hormone preparations seems to indicate that bioassay methods based on the duration of action of such preparations might be of value. The introduction into therapeutics of poly-estradiol phosphate (P.E.P.), a water soluble high molecular weight polyester of phosphoric acid and estradiol-17β (1, 2) led to the need of a method of comparing the prolonged estrogenic effect of various P.E.P.-preparations. The present paper reports on a method of bioassay based on the duration of vaginal cornification in spayed mice.

EXPERIMENTAL

Animals.—Adult female albino mice (16 to 20 gm.) were spayed two to four weeks before the start of the experiment. No priming injection was given. Following a single subcutaneous injection of the preparations to be assayed, smears were taken daily at 10 A.M. and the duration of vaginal cornification (in days) recorded in each animal. The smears were interpreted as suggested by Emmens (3); a positive smear being regarded as one which contains epithelial and cornified cells but no leucocytes.[1]

Preparations.—In addition to various batches of P.E.P.[2] synthesized under different experimental conditions, samples of estradiol benzoate and ethinyl estradiol in different vehicles were also assayed.

Design of assays.—With the exception of one six-point assay, in 26 instances a symmetrical four-point design was adopted, with two dose levels on the reference standard and the unknown preparations, respectively, and with equal spacing of log doses. The log dose interval was 0.301 in all the assays, except one, where it was 0.602. Validity

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[1] When spayed mice are smeared for 30 to 40 days, on a few occasions the smears may contain a few leucocytes in addition to cornified cells. When such smears were followed again by fully positive smears for several days, they were regarded as positive.

[2] We are indebted to AB Leo, Hälsingborg, for placing the various P.E.P. preparations at our disposal.

tests were performed in each assay, following the technique described by Gaddum (4). As an index of precision, Woolf's *L*-criterion (5) was calculated from the data of each assay. For assays depending on graded responses, b/s, *i.e.* the ratio of the regression coefficient to the standard deviation of the response provides an estimate of *L*. The justification in support of the use of this index of precision is discussed elsewhere (6, 7, 8). The relative potency and fiducial limits of error have been calculated throughout this study according to the method of Gaddum (4), with the exception that the variance of a single effect was computed by classical methods rather than from the range. This was necessitated by the relatively large number (up to 20) of animals used per dose level.

Frequency distribution of individual duration times.—This was investigated in 96 spayed mice injected with 16 μg. of a P.E.P. preparation. The accumulated frequencies showed a systematic deviation from linearity (Fig. 1a) when they were plotted on probability paper, indicating that the distribution of the individual data is not strictly normal. On the other hand, when the accumulated per cent was plotted against duration time on a logarithmic scale, a straight line was obtained (Fig. 1b). This suggested that the logarithmic transformation may be effective in normalizing the distribution of individual duration times. Furthermore, since the use of the reciprocal transformation has been recommended frequently for assays

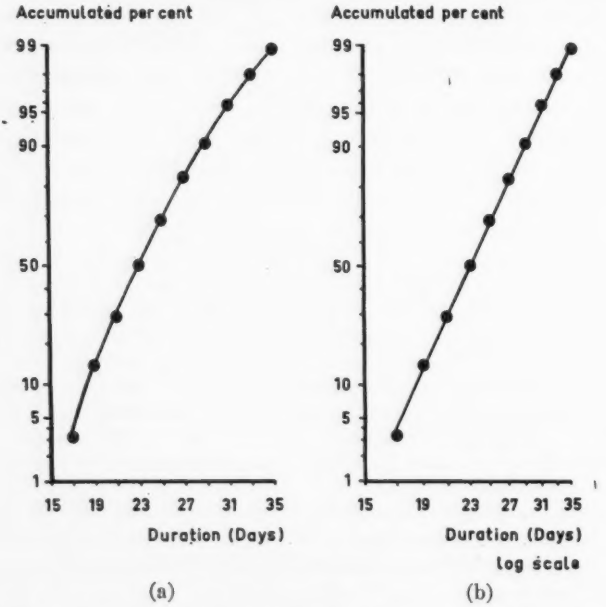

FIG. 1. Cumulative frequencies calculated from the duration of vaginal cornification in 96 spayed mice treated with a single subcutaneous injection of 16 μg. of Poly-estradiol phosphate (P.E.P.) in water. The curve on the left (Fig. 1a) indicates accumulated per cent plotted on a normal probability paper versus duration in days. The line on the right (Fig. 1b) indicates a plot of accumulated per cent versus days on a logarithmic scale.

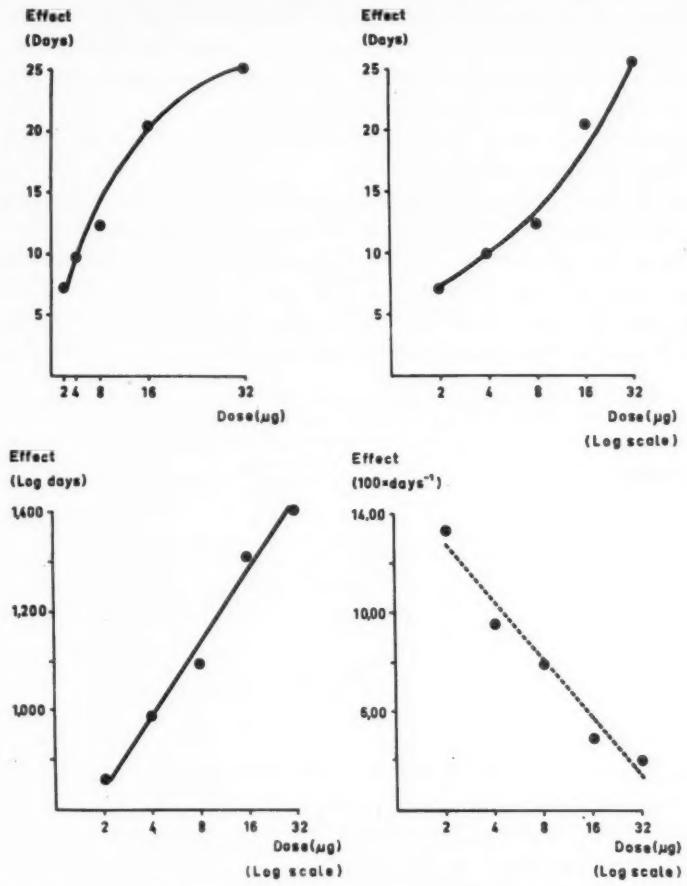

FIG. 2. Influence of different metametric transformations on the relationship between the dose of Poly-estradiol phosphate (P.E.P.) administered in water, and the duration of vaginal cornification in spayed mice. Each point corresponds to 18 animals. Dotted line indicates evidence against linearity.

based on the measurement of the duration time, this transformation has also been investigated.

The dose-response relationship.—In order to study the dose-response relationship, 5 groups of 20 spayed mice were injected with increasing doses of P.E.P. in water and the individual duration times were recorded. The data are presented in Figure 2. It should be pointed out, that a part of these data (a plot of log dose versus log days) was already presented in a previous paper (9); the data are shown here however, in order to illustrate the influence of different transformations on the dose-response relationship. It appears from the data presented in Figure 2 that the relationship is not linear

when either the arithmetic dose or the log dose is plotted against the response (duration in days). On the other hand, when the log dose is plotted against the log response, or against the reciprocal of the response,[3] an apparently linear relationship is obtained.

Statistical analysis of the data calculated with either of these transformations is shown in Table 1.

A test of significance of evidence against the hypothesis of linearity may be obtained by testing, whether the mean square depending on deviations from linearity, significantly exceeds the error mean square. Therefore the variance ratio (F) was calculated. This was found to be $F = 0.020/0.008 = 2.50$, when the logarithmic transformation was used, and $F = 0.085/0.029 = 2.94$, when the reciprocal transformation was employed. This last value

TABLE 1. ANALYSIS OF VARIANCE FOR THE DATA OF FIG. 2, USING TWO DIFFERENT TRANSFORMATIONS OF THE RESPONSE (DURATION TIME)

Transformation		x = log dose y = log days		x = log dose $y = 100 \times \text{days}^{-1}$	
Adjustment for mean		114.404		5773.6	
Source of variation	d.f.	Sum of squares	Mean square	Sum of squares	Mean square
Regression	1	3.574		1002.5	
Deviation from regression	3	0.060	0.020	25.6	8.5
Between doses	4	3.634		1028.1	
Error (within doses)	73	0.549	0.008	212.5	2.9
Total	77	4.183		1240.6	

($F = 2.94$) exceeds the allowable limit at the 5 per cent probability level (2.73). This finding would seem to cast some doubt on the advisability of using the reciprocal transformation, without a more effective investigation of its effect on the data.

In an attempt to see whether the conclusions reached with P.E.P. also apply to data obtained with other estrogens, a similar experiment was carried out, injecting increasing doses of ethinyl estradiol in oil. The dose-response lines are shown in Figure 3. Statistical analysis of the data revealed no significant evidence against linearity, either when the logarithmic-, or when the reciprocal transformation was used. Apparently, in this experiment the use of either transformation may be justifiable. When the homogeneity of variances within dose groups was checked by applying Bartlett's test (10) to the data shown in Figure 3, no significant evidence of nonhomogeneity could be obtained.

The data presented in Figure 3 indicate the usefulness of the logarithmic transformation, however, they do not exclude the reciprocal of the duration time as a response metameter.

[3] This metameter was defined as $y = 100/\text{days}$.

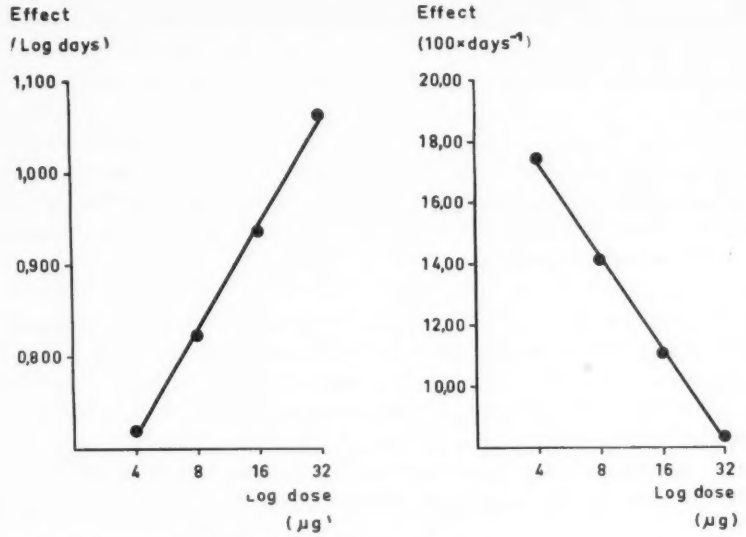

FIG. 3. Influence of different metametric transformations on the relationship between the dose of ethinyl estradiol, administered in oil, and the duration of vaginal cornification in spayed mice. Each point corresponds to 20 animals.

TABLE 2. COMPARISON OF THE EFFECT OF DIFFERENT METAMETRIC TRANSFORMATIONS OF THE RESPONSE ON THE PRECISION OF 27 ASSAYS. INDEX OF PRECISION: WOOLF'S *L*

Assay No.	No. of mice/assay	Logarithmic transformation	Reciprocal transformation
1	60	14.3	12.7
2	68	6.3	invalid assay
3	80	8.9	8.3
4	80	7.8	7.4
5	80	10.3	8.3
6	88	7.3	6.0
7	80	9.2	7.7
8	80	8.2	7.6
9	64	6.3	5.4
10	64	6.7	6.4
11	64	8.7	7.7
12	64	7.6	6.8
13	80	6.4	5.8
14	80	4.6	3.0
15	80	5.1	3.4
16	80	8.9	invalid assay
17	80	9.8	7.9
18	80	7.3	6.3
19	61	6.3	6.2
20	49	10.8	9.4
21	55	9.5	8.7
22	58	6.1	5.2
23	38	9.4	9.1
24	32	4.8	4.9
25	98	9.3	9.0
26	96	8.0	7.4
27	72	5.2	4.9
Mean (unweighted)		7.9	7.0

The precision of assays calculated with different response metameters.—Using both the logarithmic and reciprocal transformations, Woolf's L was calculated from the data of 27 assays. These values are summarized in Table 2.

The results shown in Table 2 indicate that in 26 out of 27 assays the logarithmic transformation led to higher L-values than the reciprocal transformation. This seems to indicate, that a slightly higher precision can be expected, when the logarithmic—rather than the reciprocal transformation is employed, although it would be difficult to establish this point beyond any doubt on the basis of data such as those compiled in Table 2. An inspection of the data of Table 2 also reveals that in 2 assays out of 27 the reciprocal transformation resulted in significant evidence against parallelism (assays 2 and 16), whereas the logarithmic transformation yielded valid estimates. This seems to be additional argument in favour of the use of the logarithmic rather than the reciprocal transformation.

Using the logarithmic transformation, from the data of Table 2 a mean L-value of 7.9 can be calculated. When the over-all precision of the 27

TABLE 3. POTENCY OF LONG-ACTING OESTROGENS, CALCULATED FROM 27 ASSAYS EMPLOYING POLY-ESTRADIOL PHOSPHATE (PEP) AS A STANDARD PREPARATION

Assay No.	Standard	Unknown	No. of mice/ assay	Potency of U (%)	Fiducial limits (P = 0.95)
1	PEP provisional	PEP 10 a (in water)	60	97	89 –106
2[1]	Standard (in water)	PEP 10 b (in water)	68	141	118 –171
3	Standard (in water)	PEP 18 cd (in water)	80	81	71 – 91
4	Standard (in water)	PEP 17 bb (in water)	80	56	46 – 65
5	Standard (in water)	PEP 102 ba (in water)	80	117	106 –130
6	Standard (in water)	PEP 108 de (in water)	88	100	87 –116
7	Standard (in water)	PEP 109 bb (in water)	80	93	83 –104
8	Standard (in water)	PEP 112 bd (in water)	80	92	80 –104
9	Standard (in water)	PEP 18 dd (in water)	64	77	62 – 92
10	Standard (in water)	PEP 110 id (in water)	64	87	72 –104
11	Standard (in water)	PEP 113 cd (in water)	64	90	78 –103
12	Standard (in water)	PEP 110 kh (in water)	64	94	80 –109
13	Standard (in water)	PEP 114 de (in water)	80	106	90 –125
14	Standard (in water)	PEP 114 eb (in water)	80	92	73 –116
15	Standard (in water)	PEP 110 hh (in water)	80	96	77 –118
16	Standard (in water)	PEP 18 ef (in water)	80	78	64 – 90
17	Standard (in water)	PEP 18 gh (in water)	80	83	73 – 92
18	Standard (in water)	PEP 18 ij (in water)	80	60	51 – 69
19	Standard (in water)	PEP 318 (in water)	61	103	86 –123
20	Standard (in water)	PEP 312 ga (in water)	49	103	92 –116
21	Standard (in water)	PEP 307 de (in water)	55	101	89 –115
22	Standard (in water)	PEP 315 fa (in water)	58	113	93 –140
23	Standard (in water)	PEP 301 (in water)	38	87	74 –105
24	Standard (in water)	PEP 8 bf (in oil)	32	26	17 – 38
25	Standard (in water)	EtOE[2,3] (in oil)	98	31	27 – 35
26	Standard (in water)	OEB[4] (in prop. glycol)	96	6.5	5.6– 7.4
27	PEP in prop. glycol	OEB[4] (in prop. glycol)	72	3.0	1.7– 4.2

[1] Log dose-interval = 0.602.
[2] Six-point assay.
[3] Ethinyl estradiol.
[4] Estradiolbenzoate.

assays is calculated as a weighted mean (7), a figure of $\bar{L}_w = 7.0$ is obtained. This is a satisfactory degree of precision for assays of this type (6).

The results of 27 assays (calculated with the use of the logarithmic transformation) are presented in Table 3. An inspection of the fiducial limits of error reveals, that satisfactory precision can be expected, when 60 to 80 animals are used per assay. It is of interest to note, that valid assays were obtained also when different estrogens and/or different vehicles were employed.

DISCUSSION

The drawback of the method suggested is that it is time consuming and that it requires the use of a relatively large number of animals per assay. It is however likely, that the number of animals per assay can be reduced to 40 or so, without a considerable loss in precision. The other alternative, *i.e.* the use of lower doses in order to shorten the time required for the completion of an assay, seems to be less attractive, since it may be hazardous to base assays of long-acting estrogens on duration times of less than 6 to 8 days.

It is of interest to note, that valid assays could be obtained also when different estrogens and/or different vehicles have been used. Although this may be an exception, if confirmed on different preparations, it would make it possible to carry out comparative trials on various preparations regardless of their physicochemical state (for instance crystal suspensions versus solutions).

The tentative conclusion reached in the present paper, that the logarithmic transformation might be the transformation of choice when the duration time is used as a response, is consistent with the data reported by Young (11). This worker found that the log duration time of cocaine anaesthesia was linearly related to log concentration of the drug. Also a review of earlier data (6) seems to indicate that in assays where the duration of effect is measured, plotting log time against log dose generally will yield straight lines, with approximately constant variance. It is therefore suggested, that when no previous data on the relationship between dose and duration are available, the use of the logarithmic transformation should be considered. On the other hand,—provided extremes of doses are avoided—it is likely that in the majority of assays the reciprocal transformation will also lead to similar statements as to validity and precision.

From the data reported by Meier and Tschopp (12) it would appear that the duration of effect may perhaps be regarded as an inherent property of a steroid hormone preparation, which—at least in certain cases—may not be correlated to the actual potency of the compound. If this is really the case, the assay of other long-acting hormones will also be desirable. The development of such methods seems to be well motivated for in-

stance in the androgen-field, where a considerable number of long-acting preparations have been introduced into therapeutics, whereas comparative trials on the duration of action of these preparations appear to be scanty.

SUMMARY

A method of bioassay of long-acting estrogens is described. The assay is based on the duration of vaginal cornification in spayed mice.

Twenty-seven assays were performed, using a Poly-estradiol phosphate preparation as a standard.

The data were analyzed statistically, using either the logarithmic or the reciprocal transformation of the response. In most assays both transformations led to similar statements as to validity and precision, although the precision in terms of Woolf's L was consistently higher when the logarithmic transformation was employed.

Using the logarithmic transformation, the over-all precision of the method was calculated from the data of twenty-seven assays in form of a weighted mean L. A value of $\bar{L}_w = 7.0$ was obtained.

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FURTHER STUDY ON THE ENDOCRINE RELATIONS OF CAPILLARY RESISTANCE[1]

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PREVIOUS studies have shown (1, 2, 3) that cortisone is able to restore to normal the characteristically low capillary resistance of the adrenalectomized rat, and that this effect can be antagonized by the somatotropic hormone (STH) of the anterior pituitary. Under certain experimental conditions STH was also found capable of decreasing the capillary resistance of the normal (non-adrenalectomized) rat. Subsequent observations of Wilhelmj and associates (4) corroborated this finding on the dog. STH was found to decrease capillary resistance of the normal dog and to antagonize the capillary resistance-increasing action of cortisone in the same species. Whereas in these experiments the cortisone-STH antagonism was investigated by the administration of these hormones, in the present study the same antagonism was explored from a different approach, i.e., by the surgical removal of the endocrine gland sources of the same hormones.

METHOD AND PROCEDURE

Experiments on rats. Fifty-four animals of the Sprague-Dawley strain (29 females and 25 males) weighing 150–250 gm. were hypophysectomized. Twenty-eight were subjected to bilateral adrenalectomy between the second and tenth month following hypophysectomy. In four mature albino rats (1 male and 3 females weighing 250–300 gm.) bilateral adrenalectomy was performed. Two months later the pituitary was removed. All animals were sustained on Purina Dog Chow which was enriched with meat and milk. After adrenalectomy the rats routinely received 1% NaCl for drinking water. The doubly operated rats were given 0.5 mg. desoxycorticosterone every second day. Some animals required short courses of cortisone treatment occasionally.

Experiments on dogs. Eight mature mongrels, three females and five males, weighing 8–15 kg., were hypophysectomized by the transpalatal approach. In three of these animals bilateral adrenalectomy was performed in one dog 4 months, and in two dogs 10 months following the hypophysectomy. The animals received 25–50 mg. cortisone i.m. prior to the operation and decreasing doses for a few days following it. Thereupon 1–1.5 mg. desoxycorticosterone was given i.m. every second day routinely. One per cent NaCl served for drinking water. Three mongrel dogs (1 female and 2 males of the same weight range) were first adrenalectomized and kept alive on a regime as described above.

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After a period of 35–60 days the pituitary was removed. The animals were prepared with cortisone for this second operation in a similar way as the hypophysectomized animals to the adrenalectomy.

In all experiments initial control values of capillary resistance were determined daily for one week prior to operative intervention. Subsequent readings were made every second to third day or, with critical periods, every day throughout the experiments. The negative pressure method was used for testing capillary resistance as described previously (1, 5). In the evaluation of the results, any occasionally given treatment had to be considered. No capillary resistance value was looked upon as valid unless at least two weeks had elapsed since the last cortisone injection. Since desoxycorticosterone has no effect on the capillary resistance, any treatment with this compound was regarded as not interfering with the results.

RESULTS

Effect of hypophysectomy on the capillary resistance of rats. Following the operation capillary resistance at first remained at normal levels (about 70 cm. Hg) in all 54 rats observed, then, with the exception of six rats, it began to decrease gradually between the 8th and 28th day following surgery with the males showing an earlier drop than the females. The degree of decrease in capillary resistance varied from 35 to 65 cm. Hg. The values of capillary resistance then returned to normal levels. A marked decrease in capillary resistance was accompanied by a more prolonged recovery. *Four to six weeks after hypophysectomy capillary resistance was at entirely normal values and remained so throughout our observation.* In contrast to this average behavior, no substantial change was found in the capillary resistance of six rats (all females) during the entire period of observation.

Effect of adrenalectomy on the capillary resistance in previously hypophysectomized rats. In the 13 rats that survived adrenalectomy long enough to be observed (1–2 months) capillary resistance remained normal the first week, began decreasing the second week, the third week reached its lowest level, and two weeks later approached normal values. Subsequent observations revealed a fluctuating pattern with intermittent periods of high or very low capillary resistance. Low values were accompanied by weakness and marked loss of weight. Administration of cortisone relieved these clinical signs and restored capillary resistance to normal. *It was striking that capillary resistance often remained high even when it could no longer be attributable to the previous cortisone treatment.*

Effect of hypophysectomy on the capillary resistance in previously adrenalectomized rats. Only three rats survived the second operation long enough (30, 60 and 118 days respectively) to render satisfactory observation possible. The capillary resistance had been at low levels (4–8 cm. Hg) during the entire two months between adrenalectomy and hypophysectomy. After hypophysectomy capillary resistance remained low for two to three weeks then it gradually increased during the next two weeks and subsequently showed wide fluctuations. Intermittent treatment with cortisone was necessary to prevent or counteract adrenal crisis. Nevertheless, *during several*

cortisone-free periods capillary resistance was found to fluctuate from low levels to values between 30–60 cm. Hg.

The semi-schematic Figure 1 will facilitate visualization of all the experiments on rats.

Effect of hypophysectomy on the capillary resistance of dogs. The results were unanimous in all eight experiments. During the first 3–4 postoperative days capillary resistance increased by 15–25 cm. Hg then it returned to the preoperative level, in one case after a previous drop below that level. These normal values were then maintained during the entire observation period (up to 11 months).

Effect of adrenalectomy on the capillary resistance of previously hypophysectomized dogs. Whereas adrenalectomy in the normal dog invariably decreased capillary resistance by an average of 15–25 cm. Hg, no significant change occurred in the capillary resistance of the three hypophysectomized dogs following adrenalectomy.

Effect of hypophysectomy on the capillary resistance of previously adrenalectomized dogs. The results on all three animals were unequivocal. Capillary resistance decreased following adrenalectomy and remained at low levels during the postoperative period (35–60 days). After hypophysectomy capillary resistance soon began to rise to values observed prior to adrenalectomy. In two dogs this occurred in one week, in the third dog only

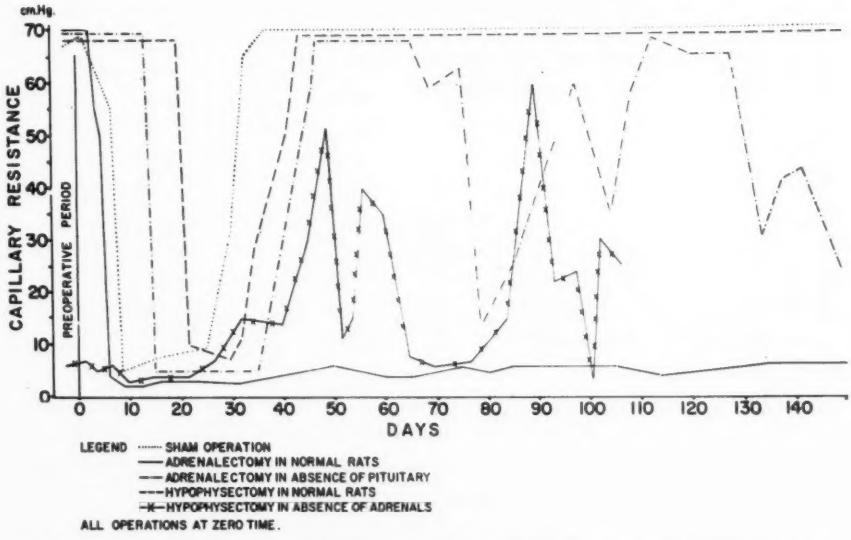

FIG. 1. Semi-schematic patterns of capillary resistance in the rat following sham operation, adrenalectomy, hypophysectomy and combined operation. Note the long "period of latency" after adrenalectomy in the hypophysectomized animal and the instability of capillary resistance following removal of both pituitary and adrenal glands.

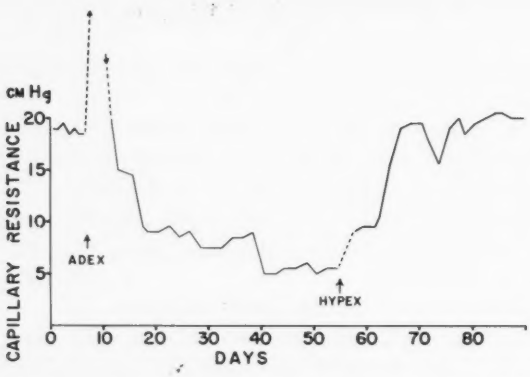

FIG. 2. Typical changes in the capillary resistance of a dog following successive adrenalectomy and hypophysectomy. Dotted lines indicate capillary resistance during cortisone treatment.

after 30 days. Figure 2 gives the detailed experiment on one of these dogs. The semi-schematic Figure 3 summarizes the results of all the experiments on dogs.

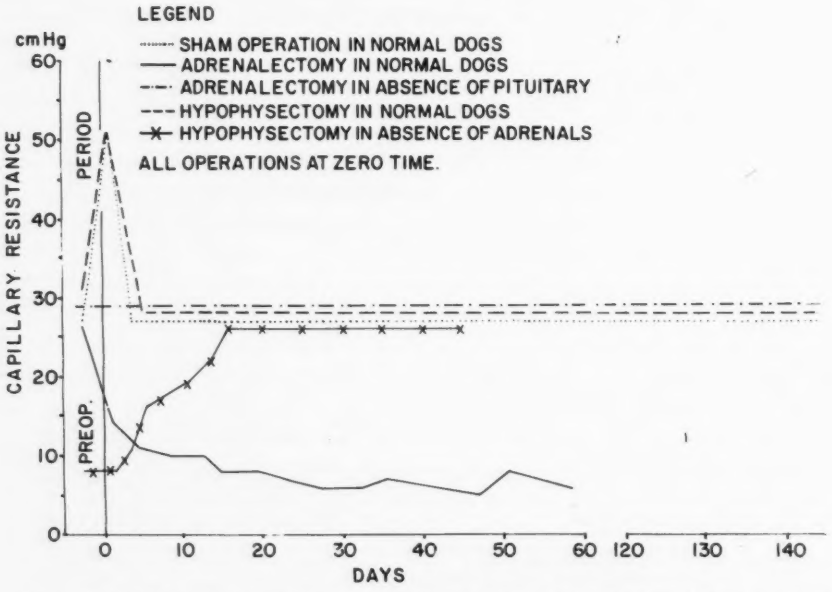

FIG. 3. Semi-schematic patterns of capillary resistance in the dog following sham operation, adrenalectomy, hypophysectomy and combined operation. Note the lack of decrease of capillary resistance of hypophysectomized dog after adrenalectomy and the return of low capillary resistance of the adrenalectomized dog to pre-adrenalectomy levels after hypophysectomy.

DISCUSSION

Stress of the most varied types may produce typical changes in the capillary resistance of human subjects as well as of experimental animals (1, 6, 7, 8, 9, 10, 11). The first changes of capillary resistance found in the present experiments following hypophysectomy were obviously due to the nonspecific effect of the surgical intervention. After these changes had subsided, capillary resistance returned to preoperative values in all animals and remained there during the entire period of observation (up to 11 months). Since a high degree of adrenocortical atrophy was found in all animals autopsied or adrenalectomized at later dates, the prompt development of adrenocortical hypofunction following hypophysectomy was necessarily assumed. The fact that this hypofunction was not reflected in subnormal levels of capillary resistance indicates that the *absence of the pituitary enabled the organism to maintain the normal capillary resistance despite atrophy of the adrenal cortex.*

A comparison of the effect of adrenalectomy on the capillary resistance of previously hypophysectomized rats with that of rats where hypophyses were intact revealed two differences. The first was in the length of "period of latency," the second in the behavior of the capillary resistance during the late postoperative state. As shown in a previous study, capillary resistance of the normal rat begins to decrease between the second and fifth day following bilateral adrenalectomy, attains extremely low levels at the end of the first week and remains so permanently. Many animals have been observed for a period of over one year, and their capillary resistance invariably has been found to be at very low levels. Following adrenalectomy in the previously hypophysectomized rats the length of the latent period varied from six to nineteen days which may indicate that either cortisone was depleted in these animals at a much slower rate or the target organs became more sensitive than in those without previous hypophysectomy.

The difference found in the late postoperative period seems to be a fundamental one. Contrary to all expectations, capillary resistance after having been at a low level for about 2–3 weeks, began to rise and soon attained entirely normal values. Henceforth it was characterized by a high degree of instability, low and high values alternating frequently. Hypophysectomy in the previously adrenalectomized rat resulted in a similar condition. This instability, the apparent lack of a higher control, seems to characterize the simultaneous absence of pituitary and adrenal glands in the rat. This instability of capillary resistance was not seen in the dog, hence the effect of the absence of both glands was even more conspicuous. The capillary resistance of the hypophysectomized dogs, in contrast to intact animals, failed to decrease upon adrenalectomy and the low capillary resistance of the adrenalectomized dogs was found to be restored to normal by hypophysectomy.

It was demonstrated previously that ACTH increases capillary re-

sistance, provided that the adrenal cortex is responsive. The observations reported in this paper seem to indicate another effect of the pituitary upon the capillary resistance. This effect, resulting in a decrease of capillary resistance, is not dependent on the adrenal cortex; yet it antagonizes the action of cortisone. This is borne out by the following experimental findings.

(1) Normal capillary resistance in the hypophysectomized rats and dogs despite adrenocortical hypofunction.

(2) Delayed decrease of capillary resistance in the rat and lack of decrease in the dog, after adrenalectomy when the pituitary is absent.

(3) Increase of the low capillary resistance of adrenalectomized rats and dogs by hypophysectomy.

It is a further question which product of the pituitary is responsible for this decreasing action upon the capillary resistance. Previous studies (12) as well as some current ones indicate that the individual hormones of both parts of the gland are either ineffective or they *increase* capillary resistance. There seems to be only one hormone in the pituitary which definitely decreases capillary resistance: the somatotropic hormone. Thus the results of the present study are considered as further indirect evidence for a new function of STH: it decreases capillary resistance and antagonizes cortisone in its capillary resistance increasing action.

The observations described in this paper permit a better understanding of the situation following adrenalectomy. When the condition ensuing after removal of the adrenals was found to be distinguished by a steady and permanently low level of capillary resistance, it was believed that the absence of cortisone was solely responsible. Now it is thought that the sentence should be modified to read "absence of cortisone in the presence of STH."

The "buffer-pair effect" of cortisone and STH upon capillary resistance is in conformity with the known antagonism between these two hormones with relation to inflammation. Since the chemical structural prerequisites of the anti-inflammatory activity of corticoids were found to be identical with those required for capillary activity (13), this paragon seems to be all the more remarkable.

SUMMARY

In an endeavor to integrate previous findings of the combined and antagonistic function of cortisone and STH upon capillary resistance, the effect of hypophysectomy upon the capillary resistance of the normal rat and dog was studied as well as the effect of adrenalectomy or hypophysectomy in the previously hypophysectomized or adrenalectomized animals, respectively.

The changes found during the first month following hypophysectomy in the normal animal are compatible with the "capillary stress response"

described previously. These changes may be attributed to the non-specific stress of anesthesia and surgical trauma necessarily preceding the actual removal of the gland.

After the non-specific stress response had worn off following hypophysectomy, capillary resistance returned to the previous normal level and remained there during the entire period of observation (up to 11 months) in both species. The condition following hypophysectomy in the intact animal seems to be distinguished by normal capillary resistance.

There were two differences between the change elicited by adrenalectomy in the capillary resistance of normal rats and in that of previously hypophysectomized rats: a) Though capillary resistance precipitously dropped in both instances, it did so in the hypophysectomized rat after an approximately four times longer "period of latency" than in the non-hypophysectomized animal. b) Whereas capillary resistance of non-hypophysectomized rats remained permanently at steady low levels after its precipitous drop following adrenalectomy, that of hypophysectomized rats returned, after its drop, to normal levels in about one month. During the observation period (50–150 days) this "normal level" proved to be extremely unstable with repeated wide fluctuations.

The same situation was found in the previously adrenalectomized rat following hypophysectomy. This instability and apparent lack of higher control seems to be characteristic of the simultaneous absence of the pituitary and adrenals in the rat.

Capillary resistance in the hypophysectomized dog, in contrast to normal dog, failed to decrease following adrenalectomy.

The permanently low capillary resistance of the adrenalectomized dog is increased and restored to pre-adrenalectomy levels by hypophysectomy.

The findings are considered as definite proof for the twofold action of the pituitary upon the capillary resistance. Besides the already known capillary resistance increasing effect of ACTH mediated by the adrenal cortex, the pituitary also has a capillary resistance decreasing effect. The latter antagonizes cortisone but is nevertheless independent of the adrenal cortex. The findings also suggest that this second action of the pituitary upon capillary resistance is due to the somatotropic hormone.

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A METHOD TO REDUCE THE INDIVIDUAL VARIATION IN AN ANTIDIURETIC ASSAY

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A LONG series of methods have been proposed for the assay of the antidiuretic activity of different substances. Some of them have achieved an outstanding sensitivity, as the method of Dicker (1), but it requires a complicated apparatus, which is not at hand in every laboratory. Ginsburg and Heller's procedure (2), though simpler, needs also the previous cannulation of the external jugular vein and a high percentage of animals fails to reach the rate of urine excretion which is needed to begin the assay. Crawford and Pinkham's method (3) is complicated by the determination of the freezing point. The specificity afforded by this technique is valuable in some instances but is unnecessary in standard assays. The present authors have chosen, for practical purposes, the method of Stein, Jinks and Mirsky (4), because the test substance is injected intraperitoneally and the rats need no special previous preparation; its sensitivity being, on the other hand, generally sufficient. The results are sometimes erratic, however, and the need of duplicates or of enlarging the number of animals per group makes this method open to criticism. This experiment is concerned with the search of this source of confusion.

METHOD

The assays were performed following the technique described by the original authors (4). The test animals were 96 male rats from the colony of this Institute with a mean weight of 162 gm. They were divided at random into four equal groups receiving respectively normal saline, 0.1, 0.3 and 0.9 mU. of Pitressin (Parke, Davis Co.) per 100 gm. of body weight. The index of antidiuretic activity (IAA) is expressed as the ratio V20/V60, where V20 is the volume of urine excreted during the first 20 min. after the second gavage and V60 the sum of V20 plus the volume of the following 40 min. (V40). The results were plotted against V20 and regression lines, correlation coefficients and analysis of variance and covariance of these data were calculated.

RESULTS

Table 1 shows the means and standard deviations of V20, V40 and IAA.

As it can be seen in Figure 1 the IAA and V20 are closely correlated ($r = 0.66$). Incidentally, the sensitivity of this method is confirmed since it is possible to detect the effect of 0.1 mU. of Pitressin. Table 2 shows the analysis of variance and covariance of these data. No difference is found

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TABLE 1. MEANS AND STANDARD DEVIATIONS OF V20, V40 AND IAA

Dose mu. pitressin /100 gm.	n	V20 $\pm s$	V40 $\pm s$	IAA $\pm s$
0.0	24	2.8 $\pm$1.13	4.76 $\pm$1.095	0.36 $\pm$0.0752
0.1	24	3.11 $\pm$0.824	3.67 $\pm$1.14	0.46 $\pm$0.0955
0.3	24	3.28 $\pm$0.99	2.30 $\pm$0.906	0.595 $\pm$0.1176
0.9	24	4.40 $\pm$1.735	1.46 $\pm$0.529	0.725 $\pm$0.146

among the slopes of the various groups. This is taken to mean that the sensitivity of the animals to the antidiuretic hormone is independent of the water load along the range here investigated, since the last is inversely proportional to V20. As the greatest sum of squares (Table 2) is attributable to the slope it is easy to understand why the influence of V20 may explain some unexpected results: it can be seen in Figure 1 that a dose of 0.9 mu. may give an IAA inferior to that of 0.3 mu. if the V20 of the first

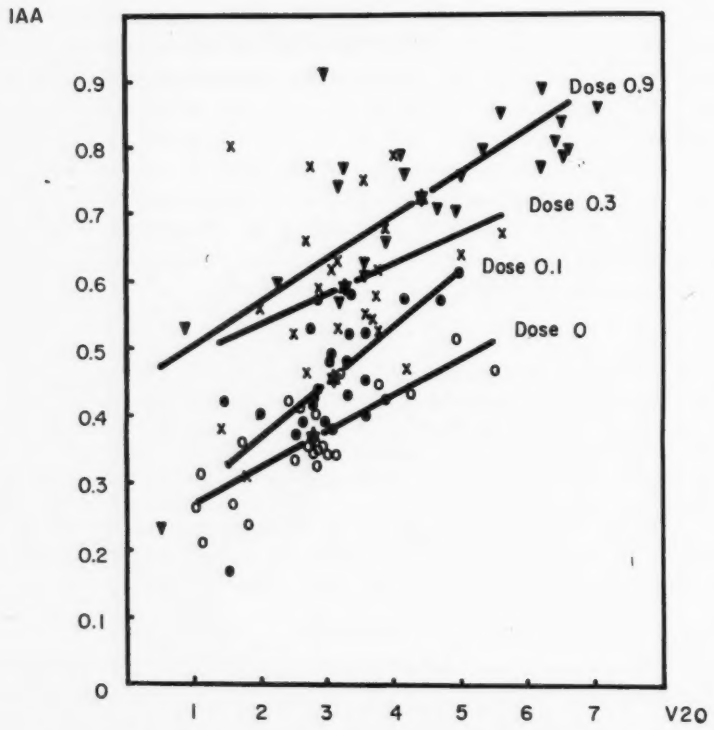

FIG. 1. These slopes were obtained from the values of IAA (V20/V60) plotted against V20 for doses ranging from 0 up to 0.9 mu. of Pitressin. There is no significant difference between them (Table 1). Note that dose 0.9 with a low V20 has a lower IAA than 0.1 with a high V20.

TABLE 2. ANALYSIS OF VARIANCE OF THE DATA OF FIGURE 1

Sources	D. of F.	Sy^2	MS	F
Doses	3	1.82	0.607	48.64
Error	92	1.148	0.01248	
Analysis of covariance of the same data				
Sources	D. of F.	Sy^2	MS	F
Slope	1	1.439	1.439	88.5
Total deviation from the slope	94	1.529	0.01627	
Doses	3	0.886	0.2953	41.8
Indiv. slopes	3	0.019	0.00633	0.89
Error	88	0.624	0.00709	

is from 2 to 3 ml. while that of the second is from 4 to 5 ml. Small groups with such distributions of V20 may very easily arise by chance and the actual effect of the dose will be obscured in consequence. This trouble can be removed by a regression, which permits to express the IAA on the basis of a common V20. These estimated data are used later for the calculation of the potency ratio with the method of Bliss (5). An alternative solution, also leading to avoid the influence of V20, is the use of V40 as the only measure of antidiuretic activity instead of the IAA's. It has been found (Table 3) that it affords a greater precision, the F of doses increasing from 41.8 up to 57.0.

TABLE 3. ANALYSIS OF VARIANCE OF V40

Sources	D. of F.	Sy^2	MS	F
Doses	3	152.9	51	57
Error	92	82.34	0.896	

DISCUSSION

The problem posed by the influence of V20 can be handled in three ways: a) by choosing a narrow range of V20, b) by adjusting the IAA to a common V20 and c) by the use of V40 alone. The latter two are better because a reduction of the range of V20 leading to the same increase of precision would make this method impractical since no departure from a given value of V20 could be accepted and, accordingly, an enormous amount of animals should be discarded. On the other hand, no further increase of sensitivity would be thus attained. Now, when the index by itself is not necessary (to compare with the original method, for instance) V40 is obviously better, because it affords a greater precision without the need to calculate the regression of it with V20 since no significant correlation between these two variables has been found.

In this experiment the significance of the difference among doses (see analysis of variance, Table 2) is partly attributable to the influence of V20, because the mean V20 of each group increases, by chance, with dose, but the converse situation could also be expected. Since the actual differ-

ence among doses is unaffected by adjusting the IAA to a common V20, the increase of precision thus obtained can be measured by the reduction of the mean square of error after the sum of squares of slope has been segregated, that is 43% (6). A further and apparently unbiased increase of significance can be obtained by the use of V40, instead of the "corrected" IAA.

On mathematical grounds it can be shown that this is a case of the so called "spurious correlation" of Pearson, Yule and Kendall (7), which happens when the same element is repeated in the y and in the x axes, i.e.: $x=X_1$, $y=X_1/X_1+X_2$, where X_1 stands for V20 and X_1+X_2 for V60.

In this case

$$r = \frac{\dfrac{M_2}{M_1} - r_{12}\dfrac{s_2}{s_1}}{\sqrt{\left(\dfrac{M_2}{M_1}\right)^2 - 2r_{12}\dfrac{s_2}{s_1}\dfrac{M_2}{M_1} + \left(\dfrac{s_2}{s_1}\right)^2}} \qquad \text{(I)}$$

where M_1, M_2, s_1, s_2 and r_{12} are respectively the means, standard deviations and correlation of X_1 and X_2. As $\text{IAA}=X_1/X_1+X_2$ then $M_2/M_1 \cong 1-\text{iaa}/\text{iaa}$, being iaa the mean IAA. The experimental r_{12} is either negative or positive and systematically non significant; accordingly, it is considered an estimation of $r=0$. Since

$$X_2 = \frac{X_2}{X_1} X_1$$

when s_{X_2/X_1} is small, $s_2 \cong s_1 M_2/M_1$ (it should be equal if $s_{X_2/X_1}=0$) it follows that $(s_2/s_1)^2 \cong (1-\text{iaa}/\text{iaa})^2$ may be an acceptable approximation. In consequence (I) may be expressed thus:

$$r = \frac{\dfrac{1-\text{iaa}}{\text{iaa}}}{\sqrt{\left(\dfrac{1-\text{iaa}}{\text{iaa}}\right)^2 + \left(\dfrac{1-\text{iaa}}{\text{iaa}}\right)^2}} = \frac{1}{\sqrt{2}} = 0.707.$$

This is the predictable correlation of IAA with V20, based on the preceding assumptions; the experimental one, arising from 196 assays, has been $r=0.687$. It follows that the influence of V20 must be removed either statistically or simply by omitting it in the index, that is, by reducing it to V40.

SUMMARY

The index of antidiuretic activity obtained by dividing the quantity of urine excreted during a period of 20 min. (V20) prior to the injection of an antidiuretic substance by the sum of this same quantity plus that of the following 40 min. (V40) is highly correlated with the first of these values

(V20), which is taken as control. It has been found that some erratic responses are attributable to this fact.

The mean square of error may be reduced by about 43% if the indexes are adjusted to a common V20. An even greater precision can be simply obtained by omitting V20, i.e. by using V40 instead of the index.

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HORMONAL EFFECTS ON SULFANILAMIDE ACETYLATION[1,2]

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EVIDENCE is accumulating which indicates that hormones influence coenzyme A dependent reactions. Androgens and adrenocorticoids in some cases have been shown to affect the synthesis of citric acid in various organs under *in vivo* (2, 3) and *in vitro* (4, 5, 6) conditions. Fatty acid and acetoacetate synthesis from acetate appear to be under hormonal control (4, 7, 8). Hormonal effects on p-aminobenzoic acid acetylation (9) and choline acetylation (10, 11, 12, 13) have also been described. Little insight into the manner in which these effects are exerted has been obtained.

This study was undertaken to explore the possibility that sulfanilamide acetylation could be a mirror reflecting hormonal influences on coenzyme A dependent reactions.

METHODS AND MATERIALS

The *in vitro* sulfanilamide acetylating system used was that of Lipmann (16). The system was incubated with gentle shaking for two hours at 37° C, deproteinized with 5% trichloroacetic acid and aliquots analyzed before and after acid hydrolysis by the method of Bratton and Marshall (17). The difference between sulfanilamide values before and after hydrolysis represented the sulfanilamide acetylated. Results are reported in per cent of control. Although a variation of a few per cent occurred from batch to batch of enzyme extract, the usual amount of acetylation in the control tubes was 55% of the 67.5 μg. of sulfonilamide present.

All steroids were introduced into the *in vitro* system as a creamy suspension obtained by oscillating the steroid with water or buffer in a 9-Kilocycle Magnetostriction Oscillator (Raytheon Manufacturing Co., Waltham, Mass. Model-S102A).[4]

Sexually mature Sprague-Dawley albino rats were injected intraperitoneally with a sterile physiological saline solution containing 5 mg. of sulfanilamide per cc. at the rate of 2 cc. per hour until a dose of 70 mg. per kg. had been given. Urine was collected under toluene for 24 hours following the final injection. After suction filtration and appropriate dilution, 5 cc. of 4N HCl per 100 cc. diluted volume were added and duplicate aliquots

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[2] A preliminary report of this work has been presented (1).

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[4] Used through the kindness of Dr. R. Kallio, Department of Bacteriology, State University of Iowa.

analyzed for sulfanilamide before and after hydrolysis. The ratio of the amount acetylated to the total amount recovered in the urine multiplied by 100 defined the "per cent acetylation." Recovery of injected sulfanilamide in the urine was 80–95%.

In the studies on testosterone injected females, 0.5 mg. of testosterone propionate was injected intraperitoneally daily for 13 to 57 days prior to retesting sulfanilamide acetylating capacity. In castration studies, the testes were removed 13 to 57 days prior to retesting acetylation capacity. Thus in the castration and testosterone propionate studies each animal acted as its own control.

The commercial insulin used was "Iletin Insulin Lilly" having a potency of 40 U. per cc. The electrophoretically pure insulin[5] had a potency of 20 U. per mg. The low zinc insulin contained a maximum of 0.1 per cent zinc and had a potency of 19 U. per mg. Zinc acetate was obtained from Coleman and Bell Co., epinephrine from Parke, Davis and Co., and DL-thyroxine from Organon, Inc. The ACTH was "ACTHAR" from Armour Laboratories. The somatotropin (STH) was Armour Beef Growth Hormone (Lot No. 285183). Testosterone, desoxycorticosterone acetate (DCA), estrone, estradiol-17β, and cortisone (recrystallized) from Mann Fine Chemicals, Inc. Cholesterol was a product of the Paragon Chemical Co. Ergosterol was obtained from Nutritional Biochemical Corp., progesterone from Bios Laboratories, Inc. and isoandrosterone from Ciba Pharmaceuticals, Inc. The testosterone propionate used for injection was "Neo-Hombreol" by Organon, Inc.

RESULTS AND DISCUSSION

The non-steroid hormones tested, with the exception of the commercial insulin and electrophoretically pure insulin preparations, had no effect on sulfanilamide acetylation *in vitro* over the concentration ranges studied

TABLE 1. EFFECT OF INSULIN AND ZINC ACETATE ON SULFANILAMIDE ACETYLATION *in vitro*

Substance added	No. of runs	Concentration*	Acetylation as per cent of control
Commercial insulin	3	4.0	76
Electrophoretically pure insulin	1	4.0	79
Low-Zinc insulin	4	4.0	106
Zinc acetate	1	2.54×10^{-4}	90
	1	5.08×10^{-4}	82
	1	7.62×10^{-4}	75

* Concentration units: insulin in units per 2.5 ml., zinc acetate in mg. per 2.5 ml.

(Tables 1 and 2). The inhibition caused by the commercial and electrophoretically pure insulins was believed to be at least partially due to their zinc content as an equivalent amount of zinc had an inhibitory effect and a low zinc insulin did not.

Of the steroids tested, testosterone, progesterone, cortisone, and DCA stimulated acetylation by more than 10% (Table 3). Dispersion of testosterone by oscillation or ether, as compared to direct addition of the

[5] We are grateful to Dr. Peter A. Tavormina (Sharpe and Dohme, Inc.) for the electrophoretically pure insulin, to Dr. Alfred Staub (Eli Lilly and Co.) for the low zinc insulin and to Dr. S. L. Steelman (Armour Laboratories) for the Armour Beef Growth Hormone.

TABLE 2. EFFECT ON NON-STEROID HORMONES OTHER THAN INSULIN ON SULFANILAMIDE ACETYLATION *in vitro*

Substance added	No. of runs	Concentration (μg. per ml.)	Acetylation as per cent of control
Epinephrine	9	0.01– 10.0	95 ±5
DL-Thyroxine	10	0.73– 73.0	100 ±2
ACTH	6	4.0 –200	94 ±3
STH	26	0.1 –1000	101 ±3

steroid to the incubation mixture, resulted in a greater stimulation being observed. Oscillation of the testosterone did not alter either the melting point, mixed melting point or infra-red spectrum of the compound.[6]

The extent to which the steroids altered the amount of acetylation was probably not limited by their concentration *per se*, but by their degree of dispersion in the aqueous system. Stimulation of acetylation by steroids dispersed by oscillation was greater at lower concentrations than by corresponding amounts of powdered steroids. After reaching a maximum, the stimulation was not increased by higher steroid concentrations.

TABLE 3. EFFECT OF VARIOUS STEROIDS UPON THE ACETYLATION OF SULFANILAMIDE *in vitro*

Substance added	No. of runs	Concentration (μg. per ml.)	Acetylation as per cent of control
Cholesterol	5	28– 840	97 ±1
Ergosterol	5	24– 760	100 ±1
Estradiol-17β	5	40–1240	99 ±0
Isoandrosterone	5	8– 240	104 ±2
Desoxycorticosterone acetate	5	20– 600	112 ±1
Cortisone	4	100–1000	114 ±2
Testosterone	6	64– 384	115 ±2
Progesterone	5	4– 80	111 ±8

Only those steroids which possess the Δ^4-3-keto moiety stimulate sulfanilamide acetylation ten per cent or more.

Preliminary studies *in vivo* indicated the suitability of a 70 mg. per kg. dosage of sulfanilamide in that (1) there could be little question of toxicity (2) most of the injected sulfanilamide was recovered, and (3) the level of acetylation rendered fluctuations in either direction readily discernible. Normal sexually mature male rats, injected with sulfanilamide at that level, acetylated 27% more than females of the same age and strain (Table 4). The greater acetylation by normal males has also been observed in rats of various ages and strains in preliminary work. No correlation between the total body weight of an animal and the degree of acetylation could be established.

Female rats injected with 0.5 mg. of testosterone propionate daily for 13

[6] Infra-red analyses through the kindness of Dr. J. P. Hummel, Department of Biochemistry, State University of Iowa.

to 57 days increased their acetylation of injected sulfanilamide 12.8% (Table 4). It was not possible on the basis of the data obtained to correlate the number of testosterone propionate injections with the increase in acetylation although the average increase after 17 to 57 injections was greater than after 13 to 17.

Castration of mature male rats had no significant effect on their ability to acetylate injected sulfanilamide (Table 4). No definite explanation can be provided for this observation. Castration at an earlier age might provide additional pertinent information, although preliminary experiments of this type have shown only a slight lowering of acetylation.

A comparison of results obtained in the *in vitro* portion of this study with those of Torda and Wolff for choline acetylation (10, 11, 12, 13) indicates

TABLE 4. EFFECT OF SEX, TESTOSTERONE PROPIONATE, AND CASTRATION ON *in vivo* SULFANILAMIDE ACETYLATION BY THE RAT

Expt. No.	No. of rats	Sex of rats	Treatment	No. of runs	Average per cent acetylation	Per cent difference	Level of significance of change
I	15	Female	———	27	49.4 ± 4.3	27.1	t = 6.69
	15	Male	———	27	68.2 ± 7.7		P < 0.01
II	13	Female	———	23	48.4 ± 4.1	12.8	t = 3.92
			Testosterone Propionate	13	54.6 ± 2.9		P < 0.01
III	13	Male	———	23	62.3 ± 8.1	1.9	t = 0.29
			Castration	13	63.5 ± 2.9		P > 0.50

no similarity of effect for those hormones studied in common. These workers found an estrogen stimulation *in vitro* of acetylcholine synthesis with a brain tissue system and an inhibition of synthesis by testosterone. It would be hazardous to draw conclusions from a comparison of two such different tissue systems as brain and liver. Hormonal interactions with various tissues of the body may be tempered by differences inherent in the enzyme systems of these tissues. A further interpretation is that the hormones studied in both the choline and sulfanilamide systems (epinephrine, thyroxine, ACTH, estrone, estradiol, testosterone, progesterone, and desoxycorticosterone acetate) do not have as a site of action any step in the formation of acetyl coenzyme A which is common to both acetylations. Rather, the action of the hormones, as has been suggested by Gurin (18), may be to direct the utilization of acetyl coenzyme A in one of several directions.

SUMMARY

In vitro addition of various hormones to a sulfanilamide acetylating system resulted in stimulation of acetylation by testosterone, progesterone,

cortisone, and desoxycorticosterone acetate. Isoandrosterone, estradiol, estrone, ergosterol, cholesterol, epinephrine, thyroxine, STH, and ACTH had little effect. Inhibition by insulin preparations could be ascribed to their zinc content.

Male rats, when injected with sulfanilamide at a 70 mg. per kg. level, acetylated 27% more than females of the same age and strain. Female rats injected with 0.5 mg. testosterone propionate daily for 13 to 57 days increased their acetylation of injected sulfanilamide 12.8%. Castration of adult male rats had no significant effect.

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INFLUENCE OF SEVERAL C_{19} STEROIDS ON THE GROWTH OF INDIVIDUAL MUSCLES OF THE GUINEA PIG[1,2]

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IN A previous report (1) castration was shown to retard the development of a number of muscles in addition to those already known (2, 3). In this study a comparison has been made of the ability of a number of C_{19} steroids to stimulate growth in the individual muscles.

METHODS

Steroids[3]: The provided steroids were further purified by chromatography on alumina and recrystallization. The identity of each steroid was confirmed by melting point and infra-red spectrum. The steroids were prepared as cylindrical pellets of approximately 15 mg. (4). The dose of each steroid was regulated by the number of pellets implanted (3).

Guinea Pigs: The guinea pigs were of the inbred Hartley (Tumblebrook Farms) strain. They were purchased in groups of 36 at approximately 8 weeks of age (350 gm. body weight). They were kept in groups of four in metal cages with wood shavings in an air-conditioned room maintained at 22–23° and with constant artificial light of 12 hours per day. The guinea pigs were fed Rockland guinea pig diet containing vitamin C and water *ad libitum;* in addition they received 30–40 gm. of carrots and a 25 mg. tablet of vitamin C twice per week. They were weighed three times per week. Animals that did not show a uniform rate of growth were discarded. In the first series of experiments, the guinea pigs were castrated at 510 (450–580) gm. body weight and the steroid pellets were implanted subcutaneously exactly 30 days later (Table 1). In all of the subsequent experiments, the guinea pigs were castrated at 510 (490–530) gm. body weight and the steroid pellets were implanted at a body weight of 610–640 gm. which was attained 30 (24–38) days later. The implantation of the individual steroids and the different doses were randomized among the different lots of guinea pigs as they reached the selected body weight. After 21 days, the animals were anesthetized with dial-urethane[4] and then bled until dead by cutting the blood vessels of the neck or the heart. The internal organs were removed and weighed on appropriate Roller-Smith torsion balances. The carcasses were sealed in separate plastic bags and frozen until the muscles were dissected. Since in the previous study (1) good agreement was obtained between the mus-

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[2] Jane Austin, Don Cockrell and Louise Howell provided valuable assistance in this study.

[3] The steroids were kindly provided by Syntex, S. A. and Ciba Pharmaceutical Products, Inc.

[4] The dial-urethane was generously provided by Ciba Pharmaceutical Products, Inc.

TABLE 1. COMPARISON OF THE EFFECT OF VARIOUS C_{19} STEROIDS ON THE BODY, CARCASS AND TOTAL MUSCLE OF THE CASTRATED MALE GUINEA PIG[1]

Steroid	Guinea pigs no.	Steroid		Body weight		Carcass, gm. %[4]	Total muscle, gm. %[4]
		Pellets No.	Absorbed mg./21d	Initial, gm.	Change[3] %		
Controls[1]	30			621	11	(486)	(119.90)[5]
Testosterone Prop.	8	1	3.2[6]	633	14	5	7
Testosterone	8	1	5.9	627[2]	13	6	13
	7	1	5.4	620	17	6	9
	8	2	9.5	627	14	3	7
	8	6	28.4	631	16	10	14
17-Methyltestosterone	8	1	7.0	611[2]	16	3	6
	8	2	10.2	626	17	6	8
	8	6	27.8	625	17	7	9
Androstan-17β-ol-3-one	8	1	1.5	627[2]	13	7	5
	7	4	3.4	621	18	6	9
	8	8	5.5	627	17	7	12
17-Methylandrostan-17β-ol-3-one	7	4	5.1	628	17	10	−2
	7	8	8.4	623	14	7	9
Androstane-3α,17β-diol	8	1	1.1	629	10	3	−4
	8	4	3.2	634	16	6	8
	8	8	6.0	631	13	4	5
17-Methylandrostane-3α,17β-diol	8	4	5.1	621	11	0	3
	8	8	8.9	629	16	6	4
17-Methylandrostane-3β,17β-diol	8	1	0.2	597[2]	10	−1	−9
	6	12	3.5	629	13	4	4
17-Methyl-5-Androstene-3β,17β-diol	8	1	0.4	597[2]	9	−3	−7
	8	12	6.5	628	12	3	0
4-Androstene-3,17-dione	8	1	7.9	616[2]	13	2	−4
	7	3	16.3	629	13	4	5
	8	6	36.7	621	13	4	5
Androstane-3,17-dione	8	1	9.6	590[2]	16	2	4
	7	3	19.0	625	17	5	5
	9	9	39.9	628	16	4	9
Epiandrosterone	8	1	6.0	628[2]	8	4	0
	6	8	29.3	634	15	6	7
Dehydroepiandrosterone	6	8	38.0	641	13	7	8

[1] The guinea pigs were castrated at 510 (490–530) gm., 3 to 4 months old. The steroid pellets were implanted subcutaneously at 610–640 gm. which was attained at 30 (24–38) days later. Autopsy was performed 21 days after implantation of the steroids.

[2] These animals and one group of controls were castrated at 510 (450–480) gm. and the steroids were implanted exactly 30 days later.

[3] Change from respective initial values after 21 days of steroid treatment.

[4] Change from control values which are in parentheses.

[5] The total of the individual right muscles multiplied by two plus the diaphragm.

[6] Equivalent to 2.5 mg. of testosterone.

cles of both sides, only the muscles of the right side, except for the retractor penis, were used for this study.

Tissue Analyses: The moisture and nitrogen content of the individual muscles were determined as in the previous report (1).

RESULTS

Organ Weights: The weights of the adrenals, kidneys, liver, spleen and thymus were not significantly changed by any of the steroids. These results are in agreement with an earlier study (3) and the failure of castration to significantly change the weights of these organs (1, 3).

Body Weights: Most of the steroids, with a few exceptions at the lowest doses, enhanced the growth rate of the guinea pigs (Table 1). The increased rates were never very great but conformed with the decrease noted after castration (1, 3). Furthermore, only suggestive but not consistently progressive changes with dose were observable (Table 1). The maximum effect seemed to be obtained at a relatively small dose.

Carcass Weights: The carcass weights of the various treated groups of guinea pigs reflected the changes in body weight and with a few exceptions were heavier than those of the controls. The increase, however, was never very great and the differences between steroid dose levels did not permit a correlation with dose.

Total Muscle Mass: The sum of the weight of the 48 individual muscles showed definite changes as a result of steroid treatment (Table 1). No progressive increase, however, with dose was produced although several of the androgens at their respective low dose levels showed little or no ability to stimulate an increase in muscle growth. In general, the changes in total muscle mass correlated with the changes in body and carcass weights.

Controls: In the course of this study four different groups of controls were observed. The average weight of the individual muscles of these 30

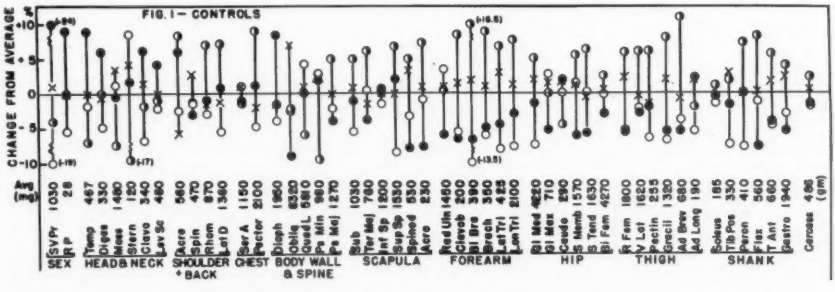

FIG. 1. *Controls:* The difference of four separate groups of control (castrated) guinea pigs from the average of all of the groups. The values are for the muscles of the right side.

Group 1: ○ (8 guinea pigs) Group 2: X (10 guinea pigs)
Group 3: ◑ (6 guinea pigs) Group 4: ● (6 guinea pigs)
See Table 1 for other details.

animals and the differences of the separate groups from the averages are presented (Fig. 1) to indicate the consistency of results among various groups. In general, the individual muscles of the different groups deviated from their respective averages by less than 10% and in most instances by less than 5%. Furthermore, the direction of the deviation was the same as that of the carcass weight. In three instances larger deviations, 13–17%, occurred.

A group of 8 animals was killed also at the time of castration. The weights of the organs and of the individual muscles and their differences from the controls were the same as previously reported (1).

Androgens: The results for all of the muscles after testosterone treatment with a single pellet in 2 separate experiments performed about 1 year apart and with 6 pellets of testosterone depict the nature of the response of the individual muscles (Fig. 2). The agreement between the two groups at the same dose level was quite good. In most instances the

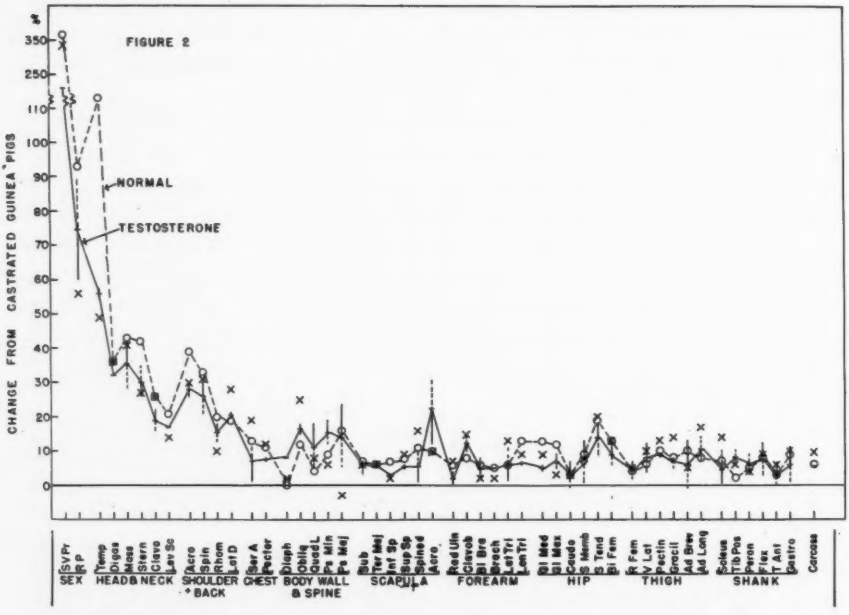

FIG. 2. The response of the individual muscles of the castrated male guinea pig to testosteron stimulaton. The values (½—½) are the averages of two separate experiments (5.9 and 5.4 mg./21 days) at approximately the same dose level. The difference from the average for the first experiment is indicated by the perpendicular solid line and the second experiment by the broken line (½). The third experiment (X) was at a five-fold increase in dose (28.4 mg./21 days). The normal values (O--O) are the averages of 12 normal guinea pigs of the same age as the castrated animals.

The differences are from the averages of all the castrated-control animals (Fig. 1). See Table 1 for other details.

values were within about 5% of each other and only in a few instances greater than 10%. The carcass (body) and all of the muscles except the temporal (*cf.* 3) were restored to or near their respective normal weights. The five-fold increase in dose produced a marked further increase in the seminal vesicles and prostates, but did not produce a consistent further increase in the growth of even those muscles that showed the greatest responses. Indeed, several of the muscles showed no greater response and some showed a smaller response.

Effect of Various Androgens on the Individual Muscles: All of the androgens stimulated the growth of one or more of the muscles to some degree. Two of the steroids, however, showed only slight activity. 17-Methyl-5-androstene-3β,17β-diol was effective at the higher dose level on only one muscle, the sternomastoid (34%), and the seminal vesicles and prostates (38%). The 17-methylandrostane-3β,17β-diol produced increases of 10–16% at the higher dose level in the clavotropezius, sternomastoid, serratus anterior, acromiotrapezius, spinotrapezius, latissimus dorsi and the internal and external obliques. The head muscles showed insignificant increases of only 7–8% and all the other muscles were in the range of the controls. The seminal vesicles and prostates were increased 95% but the retractor penis did not respond.

The other androgens were more effective and are worthy of comparison by individual muscles. The order of their potency was: 3-keto-17-hydroxysteroids with the androstanolones suggestively more efficacious than the testosterones; 3α,17β-diols; diones; and 3-hydroxy-17-ketosteroids. The presence of the 17-methyl group resulted in varying degrees of decrease in activity of the steroids.

Sex Tissues: The androgens were most effective in stimulating the growth of the retractor penis (Fig. 3). The nature of the responses with the different steroids was comparable with that produced in the seminal vesicles and prostates. The degree of response, however, was five-fold greater in the accessory sex organs (Fig. 3) which were stimulated by the highest dose of testosterone to approximately that of normal guinea pigs of the same age. Of particular interest was the reduction in potency by the introduction of the 17-methyl group in the respective steroids with the complete failure of 17-methylandrostane-3α,17β-diol to produce a significant increase in the weight of the retractor penis.

Head and Neck Muscles: The muscles of this area responded to the androgens in the same order as the retractor penis (Fig. 4). The degree of response for the respective muscles, however, was lower and differed for each muscle.

The temporal muscle showed only a slightly lower response than the retractor penis

The masseter muscle showed a further decrease except in its response to epiandrosterone, and the increase produced by dehydroepiandrosterone was not significant.

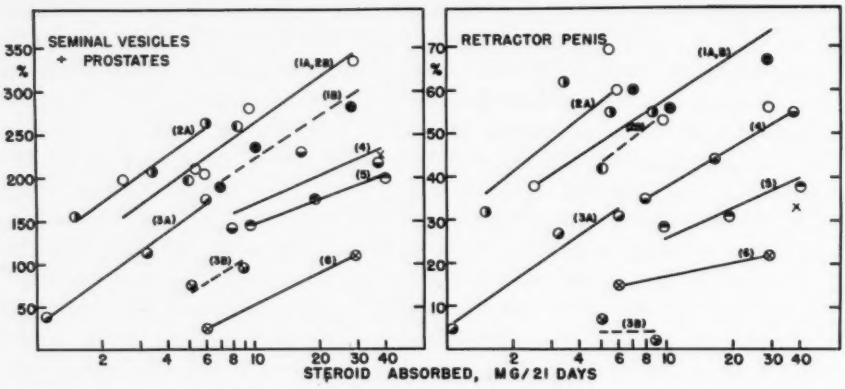

FIG. 3. *Sex Tissues:* Comparison of the ability of various steroids to stimulate growth of the retractor penis and seminal vesicles and prostates of the castrated guinea pig. See footnote Table 1 for details. The differences are in per cent of the values for the control animals (cf Fig. 1).

Curve (1): (A) testosterone ○; (B) methyltestosterone ●; *Curve (2):* (A) androstan-17β-ol-3-one ◑; (B) 17-methyl-androstan-17β-0l-3-one ◐. *Curve (3):* (A) androstane-3α,17-β-diol ◒; and (B) 17-methylandrostane-3α,17β-diol ⊗; *Curve (4):* 4-androstene-3,17-dione ◒; *Curve (5):* androstane-3,17-dione ◓; *Curve (6):* epiandrosterone ⊗; and dehydroepiandrosterone X (only one dose).

The values for normal guinea pigs at age of castration and autopsy respectively were: retractor penis, 36 and 53 mg. (93%); seminal vesicles and prostates, 2.38 and 4.80 gm. (370%).

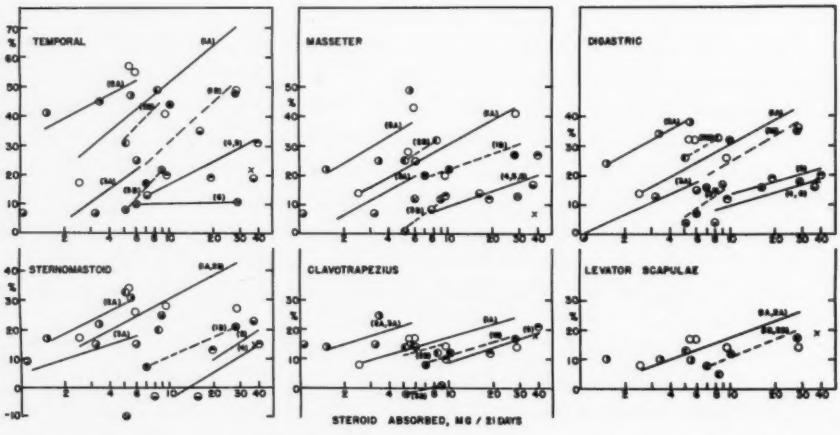

FIG. 4. Comparison of the ability of various steroids to stimulate growth of the head and neck muscles of the castrated guinea pig. See Figure 3 for details. Values of normal guinea pigs of same age: temporal, 110%; masseter, 43%; digastric, 36%; sternomastoid, 42%; clavotrapezius, 26%; levator scapulae, 21%.

The digastric responded in a manner very similar to the masseter.

The steromastoid showed roughly the same degree and nature of response to the androgens as the digastric except for the considerably decreased response to testosterone and 17-methyltestosterone at their highest dose levels. Epiandrosterone did not produce a significant increase.

The clavotrapezius showed a further decrease in response especially to the most active steroids.

The levator scapulae showed an appreciable response only to the 3-keto-17-hydroxysteroids and dehydroepiandrosterone.

Chest Muscles: The androgens were more effective in stimulating the growth of the serratus anterior than the pectorals (Fig. 5). Indeed, the pectorals showed a slight response only to the most active steroids. The

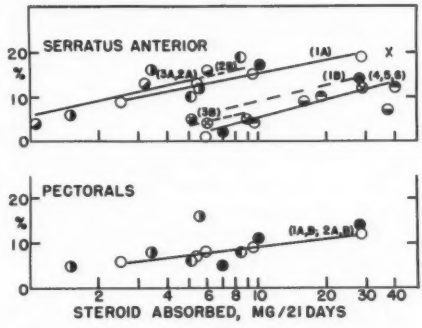

FIG. 5. Comparison of the ability of various steroids to stimulate growth of the chest muscles of the castrated guinea pig. See Figure 3 for details. Values of normal guinea pigs of the same age: pectorals, 11%; serratus anterior, 13%.

serratus anterior, on the other hand, also responded to androstane-3α,17β-diol but not its 17-methyl derivative, to dehydroepiandrosterone and also to a smaller but consistent degree at the higher doses of the diones and epiandrosterone.

Shoulder and Back Muscles: All of these muscles showed substantial responses to the 3-keto-17-hydroxysteroids and androstane-3α,17β-diol and lesser responses to the other androgens except for the rhomboideus which showed a small response and only to the 3-keto-17-hydroxysteroids (Fig. 6). Furthermore, the 17-methyl compounds were again less effective.

Other Areas: The internal and external obliques (Fig. 7) showed a substantial response to the 3-keto-17-hydroxysteroids, the diols and the diones and a questionable response to the epiandrosterones.

The adductor longus (Fig. 7) responded to a moderate degree to all of the 3-keto-17-hydroxysteroids except methyltestosterone. Among the other steroids only androstanedione produced an appreciable response which was evident at the higher dose levels.

The remainder of the muscles showed variable responses in the range of their small changes after castration (*cf.* Fig. 2).

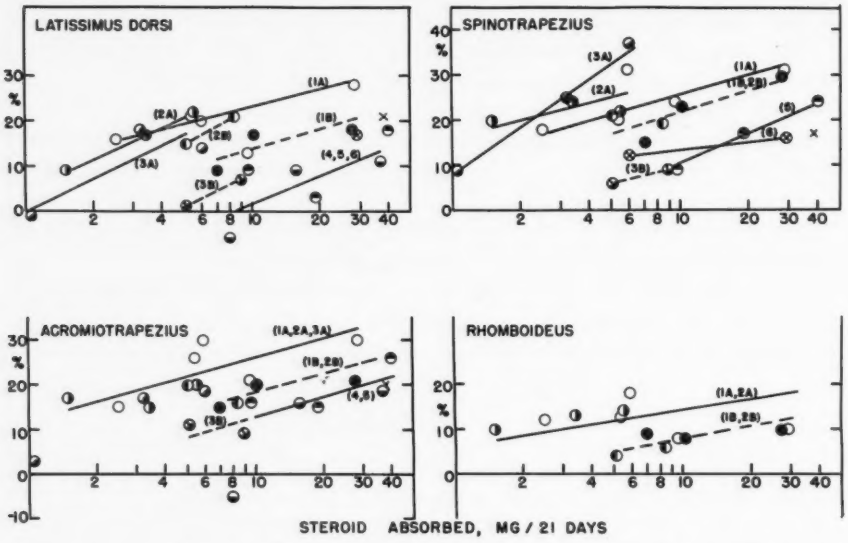

FIG. 6. Comparison of the ability of various steroids to stimulate growth of the shoulder and back muscles of the castrated guinea pig. See Figure 3 for details. Values of normal guinea pigs of the same age: latissimus dorsi, 19%; spinotrapezius, 23%; acromiotrapezius, 39%; rhomboideus, 20%.

Duration of Treatment: Since the androgens seemed unable to increase the muscles above normal size, three of the steroids were administered over a longer, 60 day, period. The prolongation of treatment at the same dose level did not enhance the response of any of the muscles or the accessory sex organs (Table 2). Indeed, the neck, shoulder and back muscles failed to show a significant response after longer treatment of the guinea

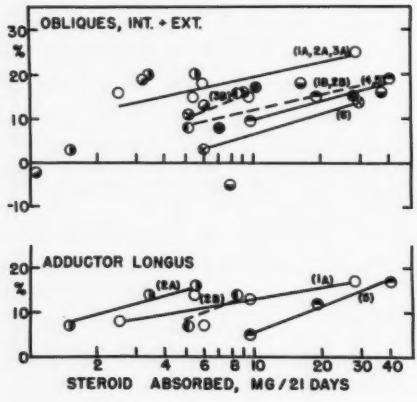

FIG. 7. Comparison of the ability of various steroids to stimulate growth of the internal and external obliques and the adductor longus of the castrated guinea pig. See Figure 3 for details. Values for normal guinea pigs of the same age: obliques, 12%; adductor longus, 8%.

TABLE 2. EFFECT OF DURATION OF ANDROGEN TREATMENT ON THE RESPONSE OF CERTAIN MUSCLES OF THE CASTRATED GUINEA PIG

	Controls		Testosterone propionate		Androstane-3α-17β-diol		Androstan-17β-ol-3-one	
Treatment, days:	21	60	21	60	21	60	21	60
Body weight, initial, gm.	621	620	633	620	634	620	627	622
Change, %	11	32	14	34	10	31	13	43
Steroid Absorbed			1.5	1.3	1.5	1.6	0.7	0.7
	Mg.	Mg.	%[1]	%[1]	%[1]	%[1]	%[1]	%[1]
Sex Tissues								
Sv. Pr.	1030	1160	196	174	112	106	156	134
Retractor Penis	28	37	38	41	27	34	32	44
Head and Neck Muscles								
Digastric	330	377	14	10	13	19	24	24
Masseter	1480	1760	14	4	15	9	22	13
Temporal	470	530	17	20	7	25	41	34
Clavotrapezius	340	415	8	6	15	8	14	13
Levator Scapulae	480	570	8	8	9	5	10	13
Sternomastoid	120	150	17	7	15	3	17	18
Chest Muscles								
Pectorals	2100	2410	6	9	11	8	5	12
Serratus Anterior	1150	1280	9	6	13	7	6	14
Shoulder and Back Muscles								
Rhomboideus	870	1070	12	3	7	1	10	9
Acromiotrapezius	560	720	15	0	17	3	17	22
Spinotrapezius	470	600	18	3	25	3	20	12
Latissimus Dorsi	1360	1620	16	8	18	8	9	15
Body Wall Muscles								
Obliques	8320	9990	16	4	19	5	3	19

[1] The changes are from the respective control values.

pigs with testosterone propionate and androstanediol. However, the greater efficacy of the androstanolone in stimulating the growth of the muscles was further emphasized.

It is of interest that in spite of castration, all of the muscles of the control animals showed further growth to the same extent as in the previous study (1).

Moisture and Nitrogen Content of the Muscles: The dry weight and moisture content of all of the muscles and the nitrogen (protein) content of the muscles of two series of experiments were determined. The per cent values for the individual muscles were as previously determined (1), therefore, only the total water and protein content of the two series of experiments are presented (Table 3). The changes in weight of the muscles were accompanied by proportionate changes in nitrogen (protein) and water.

TABLE 3. THE COMPOSITION OF THE TOTAL MUSCLES OF ANDROGEN-TREATED GUINEA PIGS[1]

Steroid	Dose, mg/21d.	Carcass, gm.	Muscle, gm.	Protein, gm.	Water, gm.	Rest, gm.
Control (8)	—	477	116.46	22.81	87.70	5.95
(10)	—	489	119.81	22.26	90.66	6.89
Testosterone	5.9	519	136.05	25.73	104.16	6.16
	28.4	531	136.32	25.77	104.10	6.45
17-Methyltestosterone	7.0	498	126.92	24.65	95.95	6.32
	27.8	521	130.43	24.26	99.22	6.95
Androstan-17β-ol-3-one	1.5	516	126.47	24.03	96.11	6.33
	5.5	517	133.94	25.66	101.98	6.30
17-Methylandrostan-17β-ol-3-one	5.1	534	117.12	24.48	86.34	6.30
Androstane-3α,17β-diol	1.1	496	115.68	23.86	86.42	5.40
	6.0	502	126.39	25.11	96.04	5.24
17-Methylandrostane-3α,17β-diol	8.9	514	124.84	24.64	94.54	5.66
Androstane-3,17-dione	9.6	495	125.09	26.00	94.79	4.30
	39.9	507	130.32	24.76	99.20	6.36
4-Androstene-3,17-dione	7.9	498	114.39	22.68	85.05	6.66
	36.7	506	125.40	23.87	95.44	6.09
Epiandrosterone	6.0	507	119.47	24.07	90.22	5.18
	29.3	514	128.10	24.60	98.11	5.39
Dehydroepiandrosterone	38.0	521	129.36	24.77	98.28	6.31
17-Methylandrostane-3β,17β-diol	0.2	476	109.55	22.34	81.88	5.33
17-Methyl-5-androstene-3β,17β-diol	0.4	468	111.86	22.29	84.89	4.68

[1] See Table 1 for details. The muscles of only two of the control groups were analysed for protein (nitrogen ×6.25) and the number of animals in the groups are in parentheses.

DISCUSSION

The response of the muscles to the various steroids conforms with the results obtained after castration. Furthermore, the relative degree of increase of the respective muscles was in proportion to the decrease observed after castration (1). It is significant, however, that none of the steroids was able to stimulate any of the castration-atrophied muscles to greater than normal size by increasing the dose or prolonging the period of treatment. The seminal vesicles and prostates, too, were restored only to normal size by the most active steroids.

The ability of the different steroids to stimulate growth of certain muscles is definitely related to chemical structure and conforms in general with previous comparisons of the physiological potencies of these compounds (*cf* 3). In addition to the main differences in potency among the steroids, some smaller but consistent variations are worthy of note. The andro-

stanolones previously had been observed to be slightly more active than the testosterones in stimulating the temporal muscle of the guinea pig (3). This same phenomenon now is apparent for a number of other muscles and is best illustrated after prolonged treatment. The introduction of the 17-methyl group, on the other hand, had been noted to decrease the activity of the various compounds towards the temporal muscle of the guinea pig (3) and now this, too, is apparent in a number of other muscles. The effect of the 17-methyl group is of special interest since its presence produces alterations in other physiological properties of the androgens (5), of which the stimulation of the production of excessive amounts of creatine (6) might be related to the myotrophic effect.

The activity of dehydroepiandrosterone and epiandrosterone are of particular interest in view of the generally accepted belief that they are very weak androgens. It should be noted, however, that these same compounds stimulated an increase in body weight of the castrated mouse out of proportion to their effect on the accessory sex organs and kidneys (6).

The variation in response of the different muscles to the same steroids probably is a reflection of the composition of the different muscles. The total nitrogen and water analyses indicate true growth. The specific nature of the growth, however, probably is different in each muscle. A detailed analysis of the protein composition and a comparison with the ability of the different muscles to respond to castration and steroid administration should be revealing. It is known that certain enzymes of the temporal muscle decrease after castration and increase after administration of a number of C_{19} steroids in proportion to the increase in muscle protein (7).

The temporal muscles showed only approximately one half the percentage stimulation observed in the previous study (3). The animals in this study were used at an older age to minimize transportation difficulties. The weights of the animals and the muscles, therefore, were proportionately greater (*cf.* 1). It is of interest that the steroids produced the same increase in abolute mass regardless of the initial mass of the muscles. The maximum effect, however, was attained at a lower dose in the older animals.

SUMMARY

Guinea pigs of the inbred Hartley strain were castrated at 510 gm. body weight and when they attained a body weight of 610–640 gm. they were implanted subcutaneously with pellets of various steroids at 1 to 3 dose levels. All of the steroids except methylandrostane-3β,17β-diol and 17-methyl-5-androstene-3β,17β-diol produced a small increase in body and carcass weight which, however, was not proportional to dose. The muscles of the head, neck, chest, shoulder, back, abdominal wall, and a few from other areas were stimulated out of proportion to the increase in body

weight while the rest increased in proportion to the increase in body weight. The ability of the steroids decreased with occasional exceptions in the following order: androstan-17β-ol-3-one; testosterone; 17-methyl-androstan-17β-ol-3-one; 17-methyltestosterone; androstane-3α,17β-diol; 17-methylandrostane-3α,17β-diol; 4-androstene-3,17-dione; androstane-3,17-dione; dehydroepiandrosterone; epiandrosterone; 17-methylandrostane-3β,17β-diol and 17-methyl-5-androstene-3β,17β-diol. The presence of the 17-methyl group decreased the activity of the steroids. The increase in weight of the muscles was accompanied by a proportionate increase in nitrogen (protein) and water.

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THE EFFECT OF HORMONES ON PHOSPHORYLASE ACTIVITY IN SKELETAL MUSCLE[1]

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CORTISONE is reported to increase glycogen storage in rabbit muscle and inhibit the activity of the enzyme phosphorylase which catalyzes reversibly the conversion of glucose-1-phosphate to glycogen (1). Epinephrine increases or maintains this enzyme activity, depending on conditions, in the isolated rat diaphragm (2). In an attempt to elucidate some unexplained interactions of these hormones in regulating muscle glycogen storage (3, 4), the following study was made on the effect of these hormones on phosphorylase activity in rat skeletal muscle.

METHODS

Muscle phosphorylase activity was determined by the method of Sutherland (2) with additional suggestions which he personally communicated. The enzyme activity in whole homogenates, prepared from muscles of well fed adult male rats, was assayed by determining the amount of glycogen synthesized from glucose-1-phosphate under standard conditions. While the rats were under nembutal anesthesia the muscle (usually the rectus femoris) was removed and quickly frozen between 2 blocks of dry ice. In some instances a small portion was used to measure the glycogen concentration. A weighed portion of the frozen muscle, approximately 300 mg., was ground in a glass homogenizer in cold 0.1 *M* sodium fluoride solution. The homogenate was transferred along with the washings of the grinder to a graduated tube and the final volume adjusted with the fluoride solution so that 1 ml. contained the equivalent of 20 mg. of fresh tissue.

The enzyme assays were made in duplicate and the results averaged. The reaction mixture for phosphorylase *a* determination contained 0.5 ml. of a 2% glycogen solution, 0.2 ml. of distilled water, 1 ml. of the muscle homogenate and 0.5 ml. of 0.064 *M* glucose-1-phosphate (adjusted to pH 6.1 with succinic acid). For total phosphorylase determination (designated as *t* hereafter) 0.2 ml. of 0.01 *M* adenosine-5-phosphoric acid solution freshly prepared was substituted for the water in the above reaction mixture. Before adding the glucose-1-phosphate, the test tubes were placed in a constant temperature bath at 37.5° C. for 8 minutes. After adding the substrate the tubes remained in the bath for 15 minutes with occasional agitation and the reaction was stopped by adding 1 ml. of cold 10% trichloracetic acid. The contents of the tubes were brought to 5 ml. with water, mixed well and filtered. To 1 ml. of the filtrate in a small centrifuge tube, 1 drop of 30% KOH and 2 ml. of 95% ethanol were added, and the glycogen was precipitated by heating and recovered by centrifugation. Glycogen was determined by the anthrone method of Seifter *et al.* (5).

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The glycogen added to accelerate the reaction (6) was subtracted from the total glycogen and the difference was the net amount of polysaccharide synthesized. Glycogen determinations in the reaction blanks showed that 98–100% of the added glycogen was recovered and it was not necessary to run blanks with each assay. The activities of phosphorylase *a* and *t* are first determined as mg. of glycogen synthesized per 1 gram of muscle in 15 minutes at 37.5° C. By dividing the activity of phosphorylase *a* by that obtained for total phosphorylase *t* (×100), an "activity ratio" is calculated which takes into consideration variations in the degree of homogenization between successive muscle samples and permits a more favorable comparison. A control muscle was always assayed concurrently with each experimental one. Since changes in phosphorylase *a* activity are important in the following study, the term phosphorylase, when used alone, designates the *a* form which does not require the addition of adenylic acid to demonstrate its activity.

RESULTS

Confirmation of the effect of epinephrine maintaining phosphorylase activity in rat diaphragms *in vitro* (2) was first made as a check on the method.

In order to establish the effect of this hormone *in vivo*, 10 μg. of epinephrine per 100 gm. b.w. was injected intraperitoneally into the rats, 30 min-

TABLE 1. EFFECT OF VARIOUS TREATMENTS ON PHOSPHORYLASE ACTIVITY RATIOS IN RAT MUSCLE

Treatment	No. of expt's	Aver. activity ratios ×100 ±S.E.		Mean differ of ratios ±S.E.		*P*-value
		Exper.	control			
		(Diaphragm)				
Epinephrine	5	78 ±5.0	51 ±4.8	27	±3.0	<.001
		(Rectus femoris)				
Epinephrine	4	86 ±3.5	69 ±2.2	17	±2.8	<.01
Cortisone	3	70 ±2.2	68 ±2.7	2	±4.4	.7
Cortisone + Epin.	4	89 ±0.8	76 ±1.2	13	±1.3	<.01
Starved 1–3 days	6	79 ±1.8	84 ±3.0	5	±2.6	>.05
Adrenalectomy	3	91 ±2.6	92 ±2.0	1	±3.9	>.8
		(Perineal muscles)				
Castration	4	92 ±4.2	92 ±1.5	0	±1.8	1.00

utes before removing the diaphragm. Consistently, a higher level of phosphorylase activity was found in each diaphragm of the treated rats when compared to the respective controls as noted in the higher activity ratios, (Table 1). In a similar experiment but using the rectus femoris muscle, epinephrine also consistently increased phosphorylase activity *in vivo* (Table 1).

Using rats in place of rabbits, an attempt was made to confirm Kerpolla's results (1) showing that cortisone inhibits phosphorylase activity. Three mg. of cortisone acetate[2] were injected daily for 6 days into the rats and the enzyme activity measured in the rectus femoris. The hormone treat-

[2] The Schering Corporation, Bloomfield, N. J., generously supplied the cortisone acetate and desoxycorticosterone used in these experiments.

ment did not affect the enzyme activity and the ratios were similar. The muscle glycogen levels of the treated rats were 115–220 mg.% higher than the controls.

The next experiments were made to test whether the antagonistic effect of cortisone on the glycogenolytic action of epinephrine (3) could be demonstrated also in altered phosphorylase activity. Rats were injected with 2 mg. of cortisone daily for 3 days (with an extra dose 12 hours before autopsy) and then injected with 10 μg. of epinephrine per 100 gm. b.w. intraperitoneally, 30 minutes before removing the leg muscles. In every case higher phosphorylase activity was observed in the treated rats when compared to the controls which received no hormone treatment. Cortisone did not prevent the effect of epinephrine in elevating enzyme activity.

The effect of adrenalectomy was also studied. Shapiro and Wertheimer (7) reported no alteration in muscle phosphorylase in rats 10–30 days after removal of the adrenals. In contrast, Stähelin and Vögtli (8) observed a decrease in muscle phosphorylase which was partially restored with desoxycorticosterone. The leg muscles of rats which had been adrenalectomized for 10 days and maintained on saline were assayed for phosphorylase. The enzyme activity was similar in the muscles of the experimental and control rats.

There was a possibility that using rats fed up to the time of autopsy might have influenced the results. Lundbaek and Goranson (9) reported that starving rats 1–4 days increased phosphorylase activity in the leg muscles. This experiment was repeated on a group of rats fasting 1–3 days. Fasting did not alter the enzyme activity in the leg muscles although the loss of glycogen averaged −261 mg.% (range −222 to −310 mg.%).

It seemed possible that the marked atrophy and loss of glycogen which occurs in the perineal muscles following castration (10) might be reflected in changes in phosphorylase activity. The perineal muscles from rats castrated 11–30 days were assayed together with control muscles of intact rats. The result showed no effect on phosphorylase in each instance even though marked atrophy of the muscles was visibly noticed.

With the exception of the effect of epinephrine, it appeared that phosphorylase activity was unaltered by the several treatments to the animal, which in some instances markedly raised or lowered the glycogen levels in the muscle. However, Cori (11) showed that electrical stimulation of a muscle for 2–3 minutes rapidly lowered phosphorylase *a* activity without effecting total phosphorylase. This suggested the possibility that the hormones might influence phosphorylase under conditions where the muscle was made to contract. In preliminary experiments it was found that direct stimulation of the rectus femoris muscle for 10 seconds with the aid of an inductorium produced a marked decrease in phosphorylase activity. Stimulation times up to 2 minutes seemed to have no greater effect. Therefore,

in the following experiments, the right rectus femoris muscle of an anesthetized (Nembutal) rat was stimulated for 10 seconds and the left muscle served as the control. The effects of various hormonal treatments on the depression of phosphorylase activity which follows electrical stimulation are shown graphically (Fig. 1). Four or more rats were used in each experiment.

In the non-treated controls, electrical stimulation depressed the activity ratio with a mean difference and standard error of 45 ± 5.9. When

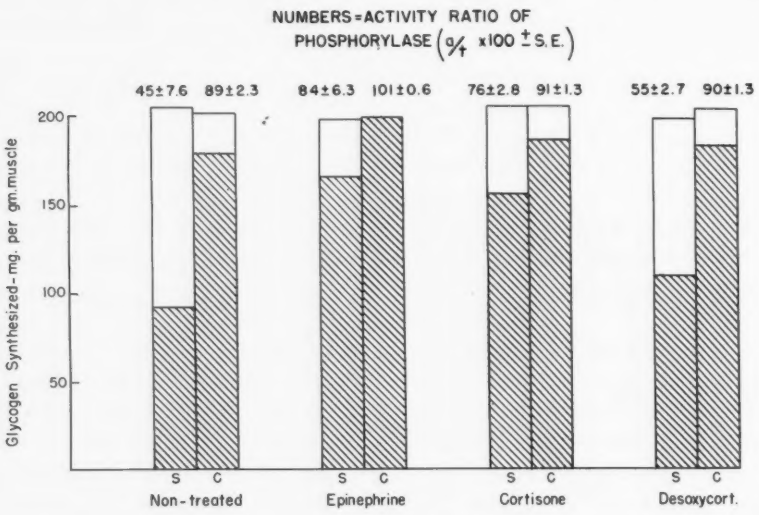

FIG. 1. Phosphorylase *a* activity (shaded portion) and total phosphorylase *t* (entire column) in the rectus femoris muscles. *S* = electrically stimulated right leg and *C* = non-stimulated control. Pretreatment of rats as indicated in text.

rats were injected with 10 μg. of epinephrine per 100 gm. b.w., 30 minutes before stimulation, the mean difference of the ratios between the stimulated and non-stimulated muscle was 16 ± 6.0, showing a marked inhibition of phosphorylase inactivation. When rats were injected with 2 mg. of cortisone daily for 3 days, the difference in the activity ratios between the two muscles was 15 ± 3.1, showing that this hormone also inhibited the inactivation of phosphorylase. When desoxycorticosterone[2] was substituted for cortisone in an experiment similar to the preceding one, electrical stimulation markedly lowered the ratio with a mean difference of 35 ± 2.0.

DISCUSSION

It is observed in Table 1 that phosphorylase activity ratios of the control rectus femoris muscles varies from 68–92 among the several experiments. Obviously if both assay reactions were permitted to reach equilibrium,

equal amounts of glycogen would be synthesized and the activity ratios would be 100. This must be avoided. However, improvement in technique can account for the above variation; the order in which the experiments were performed corresponds to the increase in the activity ratios in the controls. This in no way invalidates the results since experimental and control muscles were assayed simultaneously. The activity ratio in the diaphragm of the control differs from that in the rectus femoris but agrees with the ratio calculated from Sutherland's data (2).

Epinephrine inhibits the inactivation of muscle phosphorylase *in vitro* (2) and the muscles of epinephrine treated rats have significantly higher levels of active phosphorylase than the controls. Cori (11) has stated that all the phosphorylase in the intact resting muscle is probably in the active form but that the total phosphorylase is unaltered under conditions where the active form of the enzyme is reduced, i.e., after muscle contraction. It would seem that removal and freezing the muscle could be a sufficient stimulus to cause some loss of phosphorylase *a* and thus the activity ratios of 100 may not be attained. If epinephrine can inhibit the inactivation of phosphorylase *a* induced by a strong contraction of a muscle (Fig. 1), it may also inhibit the effects of milder stimulation which occurs during removal of the muscle. The results obtained are consistent with this concept.

Cortisone has no direct effect on phosphorylase activity in the resting muscle of the rat. The decrease in enzyme activity produced by cortisone in the rabbit (1) might be explained by the fact that this hormone causes atrophy and degeneration of rabbit muscle (12). These degenerative changes have not been reported in the rat. Neither can the inhibitory action of cortisone on the glycogenolytic action of epinephrine (3) be related to phosphorylase changes. Adrenalectomized rats maintained on salt have normal muscle phosphorylase activity and it is only when acute insufficiency symptoms and muscular weakness appear that phosphorylase activity decreases (8).

There is no correlation between the amount of glycogen and active phosphorylase in a muscle. Fasting lowered and cortisone elevated muscle glycogen with no change in phosphorylase. The effect of fasting is not in agreement with the results of others (9), although frogs starved to the point of emaciation have decreased muscle phosphorylase (11). Apparently only certain types of muscle degeneration are associated with reduced resting levels of phosphorylase activity such as denervation atrophy (13) but those which follow castration have no effect on the enzyme in the perineal muscles.

Hormonal effects on muscle phosphorylase are clearly revealed after the muscle is made to contract. The decrease in enzyme activity which follows electrical stimulation can be inhibited by pretreatment of the rats with epinephrine or cortisone but not desoxycorticosterone. Based on the generally accepted evidence that epinephrine and cortisone favor continuance

of muscular contraction, it is not inconceivable that the action of these hormones is to maintain high levels of phosphorylase activity for the continuous mobilization of glycogen for energy to meet the demands of the contracting muscle.

SUMMARY

A method for determining phosphorylase activity in whole muscle homogenates is described. Epinephrine maintained phosphorylase *a* activity in isolated rat diaphragms (confirming Sutherland) and in addition, increased the enzyme activity in the diaphragm and rectus femoris muscle when given *in vivo*. Cortisone injections did not affect phosphorylase activity and combined cortisone and epinephrine treatments resulted in the predominant effect of epinephrine. Atrophied perineal muscles of castrated male rats had normal phosphorylase activity. Fasting and adrenalectomy had no effect.

Electrical stimulation of the leg muscle for 10 seconds decreased phosphorylase activity by half. Pretreatment of the rats with either epinephrine or cortisone, but not desoxycorticosterone, inhibited the decrease in the enzyme activity which normally follows electrical stimulation.

It is concluded (1) that there is no consistent relationship between muscle glycogen concentration *per se* and muscle phosphorylase activity, (2) that the *in vivo* hormonal effects on muscle phosphorylase activity are most readily observed after the muscle is made to contract.

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STUDIES ON INSULIN ANTAGONISM IN PLASMA

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ONE group of diabetic patients, besides having hyperglycemia and glycosuria, usually develop ketosis and rapidly lose weight unless given insulin treatment. In a study of the plasma-insulin activity in these patients it has been shown that if the blood sugar is elevated at the time of the test no plasma-insulin activity is found. Moreover, when insulin is added to the plasma of these patients *in vitro*, its activity is apparently inhibited (1). When such patients are controlled, however, plasma-insulin activity is found essentially in the normal range and now the activity of added insulin is not diminished.

It seemed more likely that the inhibition reported in these patients was hormonal in origin rather than due to antibodies, as these diabetics were receiving up to but usually less than 100 units of insulin daily. This indicates no insulin resistance in the accepted sense of the term and also there were no allergic manifestations. Moreover, several severely diabetic patients had such plasma-insulin antagonism before they had ever had any insulin treatment.

In order to investigate the nature of this inhibition, normal, depancreatized, depancreatized-adrenalectomized (Long-Lukens), and depancreatized-hypophysectomized (Houssay) cats have been studied with and without various hormonal replacements.

MATERIALS AND METHODS

Cats. Operations were performed under pentobarbital anesthesia. The complete removal of pancreas, pituitary or adrenals was determined by physiological criteria and by autopsy. The principal criteria were: for pancreatectomy; the amount of glucose excreted during the post-operative fasting period (2.5 to 4.0 gm./kg./day), ketonuria, and at autopsy a fatty liver and no pancreatic tissue. For hypophysectomy, there was no gross pituitary tissue at autopsy and microscopic sections of the thyroid and adrenal glands showed the characteristic atrophy. Houssay or adrenalectomized-depancreatized animals had the usual reduction in the amount of glucose excreted during fasting and no residual or accessory tissue of the operated organs at autopsy. A single dose of penicillin at the end of operation has eliminated gross infection.

All operated animals were kept in metabolism cages and fed weighed amounts of fresh horse meat daily. Viokase[2] was added to the food of all depancreatized animals

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[2] Kindly contributed by the Viobin Corporation, Monticello, Ill.

The quantity of urinary sugar was determined daily and the presence of acetone bodies tested by the nitroprusside method.

Collection of blood samples. Under nembutal anesthesia venous blood was withdrawn into a heparinized syringe. The plasma was quickly separated by centrifugation and its glucose concentration estimated; it was then stored at 2° C and assayed for insulin activity within 8 days.

Estimation of plasma-insulin activity and of insulin antagonism. The method previously described (1, 2) was used. Briefly, the uptake of glucose from undiluted plasma is compared with the uptake from buffer solution (3) of ionic composition similar to plasma, with and without added insulin. It has been found that, if the glucose uptake is plotted against the cube root of the insulin concentration, there is an apparently linear relation over the range 10–1000 microunits/ml. which enables the plasma-insulin activity in this range to be estimated.

The slopes of such regression lines did not differ significantly although varying in position from day to day. Consequently, for each assay appropriate standards must be run each day in order to fix the position of the regression line, slope 1.154, the absence of any significant change in slope being checked from time to time. The glucose uptake from the undiluted plasma sample is compared directly with this line and the insulin activity read off.

The initial glucose level in both plasma and buffer solution is ordinarily adjusted to 300 mg.% but when the initial plasma glucose was greater than 300 mg.%, then the glucose concentration of the buffer solution was correspondingly raised. There was no significant difference in the diaphragm response to insulin over the range 300 mg.% to 550 mg.%. In all the experiments, the insulin activity of the plasma was estimated. A known amount of insulin was added to the plasma *in vitro* and the activity of the added insulin was determined. Thus, in any one experiment, the glucose uptake was determined (a) in buffer alone (called the basal level); (b) buffer+added insulin (standard); (c) plasma alone and (d) plasma+added insulin. The uptake was measured three times in each solution, 3 hemidiaphragms from different rats being used. The diaphragms in buffer and plasma corresponded, coming from the same rats, as did those in buffer +added insulin and plasma+added insulin.

The accuracy of the estimations was similar to that previously found and can be seen in the tabular presentation of the results.

Hormones used have included: cortisone acetate and hydrocortisone acetate, which were administered parenterally. The growth hormone used was prepared, assayed and generously provided by Dr. A. E. Wilhelmi (#B168GH) and by the Armour Laboratories (#M10810).

RESULTS

Table 1 shows the plasma insulin activity of normal cats. The cat's plasma is suitable for such work because the supposedly low insulin activity after an overnight fast can be measured and because the amount of insulin activity corresponds closely to that found in the morning blood samples of man with this method (2). It also agrees with the estimations of investigators who have used an *in vivo* method of assay (4, 5, 6, 7). The mean plasma-insulin activity of normal cats is 114 microunits per ml. (range 70 to 165). When insulin was added to the plasma in the amounts tabulated there was essentially complete recovery of the added insulin effect, as can be seen when the results are compared to those found with buffer plus insulin. In the right hand column the one low figures—260

microunits per ml.—represents the complete recovery of only 100 microunits added. It is clear that in the normal animal there is no inhibition or antagonism to insulin added *in vitro*.

Cats 3 and 4 had been treated with daily doses of regular insulin (Lilly) for 10 and 14 days respectively. Three days after the last dose of insulin blood was withdrawn for these assays. The purpose of this was to ascertain whether any insulin antagonism could be produced in this species within 2 weeks. No antagonism was found. These animals serve as controls for

TABLE 1. PLASMA INSULIN ACTIVITY OF NORMAL CATS

Cat. No.	Glucose uptake ±S.E.M. (mg. per 100 ml. per 10 mg. diaphragm)				Calculated insulin activity (micro units per ml.)	
	Buffer alone	Buffer + insulin[1]	Plasma alone	Plasma + insulin[2]	Plasma	Plasma + insulin
1	15.8 ±0.5	27.4 ±0.6	20.9 ±0.2	30.4 ±0.8 (1000)	120	>1000
2	11.5 ±1.5	21.8 ±0.5	16.7 ±0.8	22.3 ±0.6 (900)	165	1000
3[3]	19.3 ±0.7	35.8 ±1.4	29.3 ±0.7	34.0 ±1.2 (800)	90	1000
4[3]	15.5 ±1.1	27.1 ±1.1	—	26.5 ±1.8 (900)	—	1000
5	11.2 ±0.8	23.6 ±0.7	17.5 ±0.2	19.2 ±0.4 (100)	120	260
6	19.7 ±1.7	32.2 ±1.1	26.2 ±1.2	35.5 ±1.2 (1000)	120	>1000
7	19.6	33.1 ±1.0	26.0 ±1.6	32.7 ±1.5 (800)	70	1000

[1] Insulin was added to make 1000 micro units per ml. of buffer.
[2] Insulin added—micro units per ml. in parentheses.
[3] These animals were given insulin daily for 10–14 days (see text).

depancreatized cats which were treated with insulin for short periods before their plasma was tested. At this time interval there appears to be no immunological antagonism to insulin.

Table 2, Group A, records the results in depancreatized cats. These animals were handled in one of two ways. In some animals the blood was withdrawn 2 or 3 days after pancreatectomy and no insulin treatment. Others were maintained on adequate amounts of insulin for 5 to 10 days after operation. This permitted healing and recovery from the post-operative reaction so that when insulin was discontinued for 3 days, an uncomplicated insulin deficiency was present. Both groups (3 days post-operative and 3 days post-insulin) yielded similar results and so are placed together. The plasma of these animals had no measurable insulin activity and it

strikingly inhibited the action of insulin added *in vitro*. The "absence" of insulin effect was expected. In contrast to the 100% recovery of insulin added to normal plasma, the plasma of these depancreatized animals permitted the recovery of only 10 to 25% of the added insulin. In other words, there was a mean inhibition of added insulin of 85%.

In Groups B and C of Table 2, Houssay and Long-Lukens animals were studied. In both groups no insulin was given after pancreatectomy. In the

TABLE 2. PLASMA INSULIN ACTIVITY OF VARIOUS DEPANCREATIZED CATS

Cat. No.	Glucose uptake ±S.E.M. (mg. per 100 ml. per 10 mg. diaphragm)				Calculated insulin activity (micro units per ml.)	
	Buffer alone	Buffer + insulin[1]	Plasma alone	Plasma + insulin[1]	Plasma	Plasma + insulin
	A. Depancreatized only					
8	17.5±1.9	28.2±1.6	19.7±1.2	22.0±1.6	0	120
9	16.8±0.7	28.0±0.9	18.3±0.2	23.1±0.9	0	220
10	20.5±0.8	32.6±1.1	23.4±0.8	28.0±0.8	0	250
11	17.6±2.0	30.4±0.6	18.0±1.3	25.0±0.6	0	200
12	17.0±0.5	27.9±0.6	15.7±0.7	22.2±0.5	0	170
13	14.1±0.2	25.2±1.3	13.5±1.3	18.4±1.0	0	100
14	15.2±1.7	29.0±1.6	14.1±1.7	22.5±0.9	0	120
15	16.2±1.1	28.4±0.5	—	23.2±0.5	—	200
16	19.0±0.6	31.8±1.0	—	18.2±0.6	—	0
	B. Depancreatized: hypophysectomized					
17	15.8±0.5	28.6±1.6	15.7±1.2	27.0±0.5	0	1000
18	12.6±0.5	23.1±0.4	13.1±1.2	22.8±0.3	0	1000
19	16.6±0.7	28.0±1.2	16.8±0.9	29.4±0.7	0	1000
	C. Depancreatized: adrenalectomized[2]					
20	14.9±0.6	27.4±0.7	14.1±1.4	26.8±1.3	0	1000
21	13.1±1.6	26.1±1.6	16.1±0.9	27.8±1.3	0	1000
22	16.5±1.4	28.6±1.1	16.6±1.0	29.4±1.0	0	1000

[1] Insulin was added to make 1000 micro units per ml. of buffer or plasma.
[2] Maintained on desoxycorticosterone acetate only.

six animals of these groups the plasma assays were made 3 to 19 days after pancreatectomy, which on the average meant a much longer period without insulin. As in the simply depancreatized animals, there was no measurable insulin activity in the plasma. Unlike depancreatized cats (Group A) there was no antagonism to insulin added *in vitro* and 100% of added insulin was recovered. These results led to the attempts to restore the antagonism to insulin which are shown in Table 3. In Table 3 Groups A and B record the results in Houssay cats treated with hydrocortisone and with growth hormone respectively. In all the experiments in Table 3 the dose of growth hormone was 3 mg. per cat per day, and that of cortisone or hydrocortisone was 10 mg. per cat daily. None of these animals were given insulin after pancreatectomy and the plasma assays were made 6–18 days after pancreatectomy. Both groups gave similar results in that the plasma

TABLE 3. EFFECT OF HORMONAL TREATMENT ON INSULIN ANTAGONISM IN PLASMA

Cat. No.	Glucose uptake ±S.E.M. (mg. per 100 ml. per 10 mg. diaphragm)				Calculated insulin activity (micro units per ml.)	
	Buffer alone	Buffer + insulin[1]	Plasma alone	Plasma + insulin[1]	Plasma	Plasma + insulin
A. Depancreatized: hypophysectomized treated with hydrocortisone 4 to 9 days						
23	23.2 ±1.3	33.4 ±1.0	23.7 ±1.0	35.5 ±1.5	0	1000
24	21.4 ±1.7	35.1 ±0.4	21.8 ±0.3	35.4 ±1.4	0	1000
25	20.5 ±0.5	31.4 ±1.5	20.0 ±0.8	32.0 ±1.2	0	1000
B. Depancreatized: hypophysectomized treated with growth hormone 4 to 9 days						
26	13.1 ±0.7	25.2 ±1.0	14.4 ±0.5	27.7 ±0.6	0	1000
27	12.7 ±0.6	24.2 ±1.7	13.0 ±0.7	25.0 ±1.6	0	1000
28	14.2 ±0.9	25.2 ±0.4	13.9 ±0.6	24.7 ±0.4	0	1000
29	16.0 ±0.3	26.7 ±0.8	16.3 ±0.7	26.4 ±0.8	0	1000
C. Depancreatized: adrenalectomized[2] treated with cortisone for 2 days						
30	12.0 ±1.0	28.3 ±1.4	12.6 ±1.4	27.1 ±0.9	0	1000
31	15.9 ±0.9	30.9 ±1.6	—	32.6 ±1.6	—	1000
D. Depancreatized: adrenalectomized[2] treated with cortisone or hydrocortisone for 3 to 4 days						
32	15.6 ±1.6	27.9 ±1.2	14.8 ±1.8	23.9 ±0.8	0	190
33	22.9 ±1.0	33.5 ±0.6	24.1 ±0.4	23.9 ±1.3	0	0
34	17.7 ±1.1	28.0 ±1.5	15.8 ±0.9	17.8 ±1.6	0	0
E. Depancreatized: adrenalectomized[2] treated with growth hormone for 4 days						
35	9.3 ±0.5	21.8 ±0.9	9.8 ±0.9	21.3 ±0.8	0	1000
36	12.2 ±1.0	22.4 ±1.1	11.7 ±1.0	22.2 ±1.3	0	1000
37	16.6 ±1.6	29.3 ±1.0	15.1 ±1.8	29.4 ±1.5	0	1000

[1] Insulin was added to make 1000 microunits per ml. of buffer or plasma.
[2] Maintained on desoxycorticosterone acetate throughout experiment.

from these animals had no measurable insulin activity and showed no antagonism to insulin added *in vitro*.

In Groups C, D and E, Table 3, Long-Lukens animals were studied. None of these animals were given insulin after pancreatectomy. In these eight animals the plasma assays were made 5–9 days after pancreatectomy. There was no measurable insulin activity in the plasma from any of these cats. Group C shows that 10 mg. of cortisone daily for *2 days* does not restore the inhibiting properties of the plasma from these animals. Whereas, in Group D it can be seen that there is again inhibition of added insulin when the animals have had treatment with cortisone or hydrocortisone for *4 days*.

Group E shows the results when these animals are treated with growth hormone. Treatment with this hormone did not restore the inhibition to the plasma of these animals.

Table 4 summarizes the results. The mean glucose uptake above the

level achieved by the diaphragm in buffer alone (*i.e.* with no insulin added to the medium) has been recorded for each group. There is no significant difference in the response to insulin added to buffer in a final concentration of 1000 microunits per ml. for any of the groups. In the normal animals there is a highly significant increased uptake of glucose above zero, but in all the other operated animals, in which pancreatectomy was the common feature, the glucose uptake does not differ significantly from zero, indicating no measurable insulin activity. There is no significant difference between the glucose uptake in buffer plus insulin and plasma plus insulin (first and third columns of figures), except in the depancreatized and the depancreatized: adrenalectomized (Long-Lukens) treated with hydrocorti-

TABLE 4. SUMMARY OF EXPERIMENTS ON PLASMA INSULIN ACTIVITY AND INSULIN ANTAGONISM

Group and (No.)	Mean glucose uptake above the level of diaphragm in buffer alone (±S.E.M.) mg. per 100 ml. per 10 mg. diaphragm		
	Buffer+insulin[1]	Plasma alone	Plasma+insulin[1]
Normal (7)	12.63±0.74	6.60±0.73	13.33±0.82
Depancreatized (9)	11.73±0.50	0.57±0.48	6.19±0.47
Depan: Hypox (3)	11.60±0.68	0.20±0.09	11.10±0.75
Depan: Hypox+F[2] (3)	11.55±0.74	0.14±0.32	12.25±0.63
Depan: Hypox+GH[2] (4)	11.33±0.35	0.28±0.52	11.95±1.05
Depan: Adrex (3)	12.53±0.27	0.81±1.03	13.22±0.81
Depan: Adrex+E or F[2] (3)	11.52±0.64	−0.50±0.89	2.57±1.92
Depan: Adrex+GH[2] (3)	11.80±0.79	−0.50±0.58	11.60±0.82

[1] Insulin was added to make 1000 micro units per ml. of buffer or plasma.
[2] F = Hydrocortisone acetate.
E = Cortisone acetate.
GH = Growth hormone.

sone or cortisone. In these two groups (lines 2 and 7) there is a highly significant decrease of glucose uptake in the plasma samples as compared with the buffer, indicating the inhibition of insulin activity.

A few pilot experiments have been done to ascertain where the inhibitory factor resides in the plasma. After ultrafiltration of the plasma of depancreatized cats, the filtrate fails to inhibit added insulin, a fact which suggests that the inhibitor(s) is a protein or protein-bound substance. Moreover, when this inhibiting plasma is saturated with sodium chloride and the precipitate so formed is centrifuged, washed and redissolved in buffer solution, it inhibits the activity of added insulin. In order to control this observation, we have performed the same salting out procedure with plasma from Long-Lukens and Houssay cats and in neither case was any inhibition found in the resulting buffer solution.

Full saturation with sodium chloride apparently brings down euglobulins (mainly β and γ globulins), the pseudoglobulin remaining in solution with the albumin (8). Tentatively it would appear that the inhibition resides in the β or γ globulin fraction. The inhibition in the plasma is not affected by repeated freezing to −70° C and thawing at 37° C which strongly sug-

gests that it is not a lipoprotein. In this connection, the insulin antagonism in the plasma of patients with severe diabetic acidosis has recently been studied (9). The antagonist appears to reside in the α-globulin fraction for on electrophoresis it migrates predominantly in this fraction of the plasma proteins and is not a lipoprotein.

DISCUSSION

Excluding clinical and laboratory studies on antibodies (immunological antagonism) to insulin, antagonism to insulin has been studied in the laboratory in various ways.

Experiments on diaphragms from variously treated rats. In hypophysectomized rats, the diaphragm takes up more glucose and deposits more glycogen than normal (10, 11, 12, 13) but there was no change in the insulin response (10, 12, 13, 14). By raising the glucose concentration in the medium Stadie (15) obtained greater insulin effects with hypophysectomized rat diaphragms than with those from normal rats. Injection of growth hormone (pure or crude) or cortisone into these animals did not affect the insulin response (16).

Diaphragms from adrenalectomized rats showed no change (17) or only a slight increase (13) compared with those from normal animals and the sensitivity to insulin was also unaffected with or without prior injection of growth hormone or cortisone (16, 17, 18). However, if adrenalectomized or hypophysectomized rats are given growth hormone and cortisone together, then the insulin response of the diaphragms is reduced (16).

In all these experiments, the pancreas was intact during the pretreatment with hormone. Insulin secretion was therefore a factor in conditioning the final hormonal equilibrium of the diaphragms which were tested *in vitro.*

Experiments on normal rat diaphragms. The glucose uptake by normal diaphragms in various types of sera were studied by Bornstein and Park (19). These authors found that the serum from alloxan diabetic rats inhibited the uptake of glucose by the diaphragm. If either adrenalectomy or hypophysectomy was performed on these animals then the glucose uptake returned to normal. When growth hormone and cortisone were injected into hypophysectomized alloxan-diabetic rats then the serum from these animals again inhibited glucose uptake; neither growth hormone or cortisone would give this effect if injected alone, and neither contributed to the effect if merely added to the medium.

When insulin was added to any of these sera *in vitro* there was no decrease in insulin response. However, insulin was added to make a final concentration of 0.1 unit/ml. or 100,000 microunits/ml. On the basis of our results, this concentration is so high that any insulin antagonism would probably be obscured since it would involve the inhibition of some 800 microunits of the 100,000 microunits present.

Experiments in animals tested by the response to insulin. This subject has

been studied and reviewed by de Bodo and his associates (20, 21). In their experiments, growth hormone or cortisone abolished the insulin sensitivity of hypophysectomized dogs, yet the plasma from our cats so treated did not inhibit the action of added insulin on the rat diaphragm. Likewise, growth hormone in the absence of the adrenals (hypophysectomized: adrenalectomized dogs) abolished insulin sensitivity (21) but did not result in the antagonism of plasma to insulin in our adrenalectomized:depancreatized cats. These results are compared to illustrate the problems inherent in such studies of insulin antagonism. Aside from the difference in species there are two obvious differences in procedure. de Bodo *et al.* (20, 21) tested the response of the treated animals' tissues and not the reaction of a selected normal tissue (rat diaphragm). Secondly, our animals were depancreatized but theirs were not, so that an influence on endogenous insulin secretion could have been a factor in their results. Such differences should encourage the use of all available methods in the study of antagonism or resistance to insulin. An area of agreement between these two types of experiment may well exist. de Bodo *et al.* (20, 21), Bornstein and Park (19), and others agree that the use of growth hormone and cortisone together is the most strongly diabetogenic and insulin-antagonistic combination. We have not tested both hormones together, but the inference from our results is that the presence of both hormones is required if the plasma of the treated animal is to inhibit the action of insulin on the normal rat diaphragm.

SUMMARY

Insulin antagonism in plasma and plasma-insulin activity has been studied in normal, depancreatized, Houssay and Long-Lukens cats with and without hormonal replacement. The mean plasma-insulin activity in normal fasting cats was 114 microunits/ml. and there was no inhibition of insulin added *in vitro*. In the plasma of depancreatized cats there was no recoverable insulin activity. The inhibition of added insulin was indicated by the fact that of 1000 microunits/ml. added, only 15% was recovered. This inhibition within plasma of depancreatized cats was removed either by hypophysectomy or adrenalectomy. In either Houssay or Long-Lukens cats there was again no plasma insulin activity, but now the activity of insulin added with plasma was fully recovered. Treatment with cortisone or hydrocortisone for four days restored the inhibiting properties of the plasma of Long-Lukens animals. The same treatment for two days did not restore this inhibition. Treatment with hydrocortisone for four to nine days did not restore the inhibition to the plasma from Houssay animals. Growth hormone injected for four days into either Houssay or Long-Lukens animals also failed to restore the inhibiting properties to the plasma.

It is therefore concluded that the insulin antagonism found in the plasma

of depancreatized cats is the result of the combined activity of the pituitary and adrenal cortical oxysteroids. Preliminary observations indicate that this antagonism resides within the alpha, beta or gamma globulin fraction of the plasma-proteins but is not a lipoprotein. This is also apparently the case with the antagonism to insulin previously reported within plasma of uncontrolled insulin-requiring diabetic patients.

Acknowledgments

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ALTERATIONS IN THE RAT ADRENAL CORTEX INDUCED BY SODIUM DEFICIENCY: STEROID HORMONE SECRETION[1]

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RECENT investigation has shown that restriction of sodium intake results in altered hormone secretion by the adrenal cortex. Luetscher and Axelrad (1) demonstrated in normal men that following sodium deprivation the urinary excretion of aldosterone was increased but that there were no significant changes in the excretion of other adrenal hormones. These experiments were of short duration and studies of the effects of prolonged sodium restriction on adrenocortical hormone secretion have not been reported.

The purpose of the investigation described herein was to evaluate the effect of severe, chronic sodium deficiency on the secretion of steroid hormones by the rat adrenal cortex. It has been demonstrated that prolonged sodium depletion results in increased production of aldosterone but that the secretion of other adrenocortical hormones is greatly diminished. Histologic studies were also conducted and are presented in the accompanying publication (2).

MATERIALS AND METHODS

Young, male rats of the Wistar strain weighing approximately 60 gm. were fed a sodium deficient, synthetic diet which was otherwise nutritionally adequate. Control animals received the same diet to which was added 0.24% sodium (as sodium chloride) and were pair-fed with the deficient group. The diets are completely described in the accompanying paper (2). Equal numbers of animals were selected randomly from each group and sacrificed by decapitation 2, 4, 7, 9, 14, 30, and 60 days after beginning the experiment. At the time each rat was sacrificed, the serum sodium and potassium, blood hematocrit, body and adrenal weights were determined. The right adrenal from each animal was fixed and suitably prepared for histologic examination. The left adrenal was weighed, bisected, and placed in 2 ml. of Krebs-Ringer-phosphate-glucose solution, pH 7.4. One unit of ACTH was added to the medium and the mixture was incubated aerobically for 2 hours at 37° C. in a Dubnoff metabolic incubator. On completion of incubation, the steroid hormones secreted into the medium were recovered and quantitatively determined by a method which has been previously described (3). This procedure utilizes a spectrophotometric technique which measures all steroid compounds possessing an α, β, unsaturated ketone structure in ring A of the molecule.

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After total adrenal hormone secretion was determined, the individual steroids were separated by the paper chromatographic method of Zaffaroni (4). This analysis was performed by placing on the chromatograms the combined adrenal secretion of all sodium-deficient animals sacrificed at each time interval. Paper chromatographic resolution of hormones secreted by the adrenals of control rats was accomplished in the same manner. Identification of aldosterone as one of the compounds secreted by the isolated rat adrenal was achieved by use of the method of Neher and Wettstein (5).

RESULTS

The rats fed the sodium deficient diet gained weight throughout the period of observation but at a rate which was significantly[2] slower than that of pair-fed controls (Fig. 1). Although the average body weights of

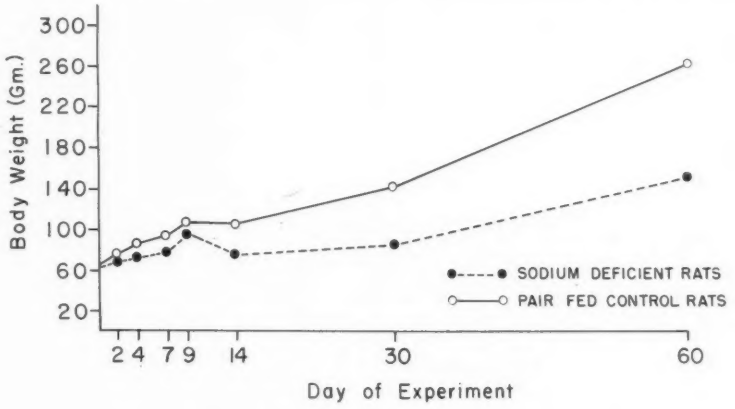

FIG. 1. Average body weights of experimental animals.

deficient animals were less than that of controls at each time interval, mean adrenal weights for the two groups were almost the same (Table 1). When adrenal weight was related to body weight, therefore, significant adrenal hypertrophy was demonstrated in animals depleted of sodium for two weeks or more (Table 1).

In rats fed the sodium-deficient diet there was a decrease in serum sodium concentration and an increase in serum potassium and blood hematocrit when compared to control animals (Fig. 2). The sodium level of the deficient group declined progressively throughout the period of observation. The elevation in potassium concentration occurred early and remained at about the same level for the duration of the experiment. The blood hematocrit values also were elevated during most of the experimental period. The alterations in serum electrolytes and blood hematocrit which occurred in the sodium depleted animals were statistically significant.

[2] Observations which are considered significant have a P value = <0.01. Statistical analyses included linear regression and the analysis of variance (6).

TABLE 1. MEAN ADRENAL WEIGHTS OF SODIUM DEFICIENT AND CONTROL RATS AT VARIOUS INTERVALS AFTER BEGINNING EXPERIMENTAL DIETS

	Duration of sodium deficiency	Number of animals	Weight left adrenal (mg.)	Weight left adrenal (mg.) per 100 gm. body weight
Deficient	2 days	5	8.2	11.7
Control		5	7.8	10.4
Deficient	4 days	5	8.3	11.3
Control		5	8.6	10.0
Deficient	7 days	5	9.2	11.5
Control		5	10.0	10.7
Deficient	9 days	5	9.5	10.0
Control		5	9.2	8.6
Deficient	14 days	10	8.9	11.6
Control		10	8.2	7.8
Deficient	30 days	10	10.3	11.8
Control		10	11.3	7.9
Deficient	60 days	6	15.3	9.9
Control		6	17.0	6.4

Steroid secretion by the isolated adrenals of deficient rats and pair fed controls is presented in Figure 3. Total adrenal hormone secretion of control rats increased progressively as the animals grew. In sodium deficient animals, however, steroid hormone secretion continued at a relatively con-

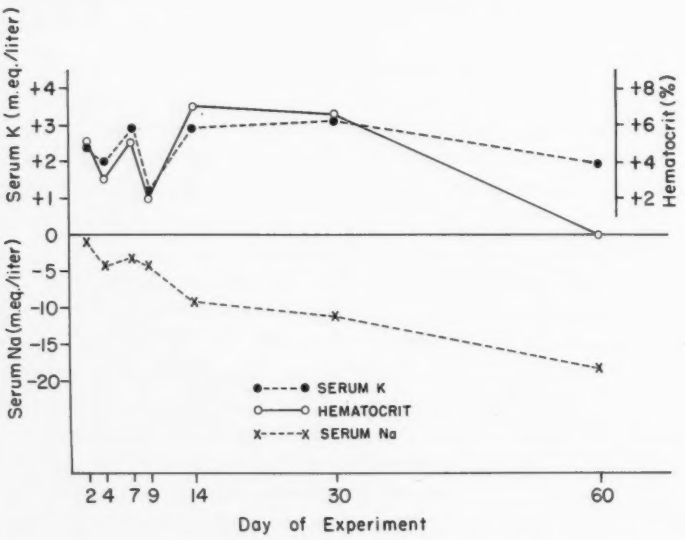

FIG. 2. Serum sodium and potassium and blood hematocrit of sodium deficient animals compared with that of pair fed controls.

stant and significantly lower rate throughout the experiment despite the fact that the adrenal glands became hypertrophic.

Previous investigations have shown that isolated adrenals of normal rats secrete four steroids when stimulated by ACTH and that corticosterone is secreted in greatest quantity (3, 7). Paper chromatographic separation of the hormones secreted by the adrenals of control rats in this study similarly revealed the presence of four compounds throughout the experimental period (Fig. 4). Chromatographic analysis of the adrenal secretion of sodium-deficient animals on the second and fourth day also revealed a normal pat-

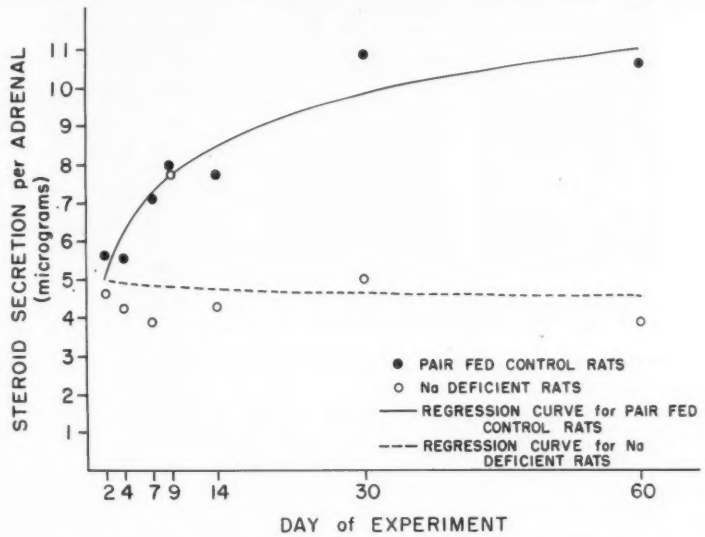

FIG. 3. Total steroid hormone secretion by the isolated adrenals of experimental animals.

tern of steroid production. By the ninth day, however, the chromatograms demonstrated that these adrenals secreted steroid 2 in increased amount. Progressively larger quantities of this compound were present on chromatograms of the adrenal secretion of sodium depleted animals on days 14, 30, and 60 (Fig. 4). This steroid was eluted and identified as aldosterone. The chromatograms also demonstrate that while aldosterone secretion became greater with increased duration of sodium depletion, the secretion of steroids 3 and 4 was greatly diminished. Previous investigation has established that steroid 4 is corticosterone but the identity of steroid 3 has not been elucidated (7).

DISCUSSION

In this investigation it has been demonstrated that aldosterone secretion by the isolated rat adrenal gland is increased as a result of sodium deficiency. This finding is in agreement with the observations of Leutscher

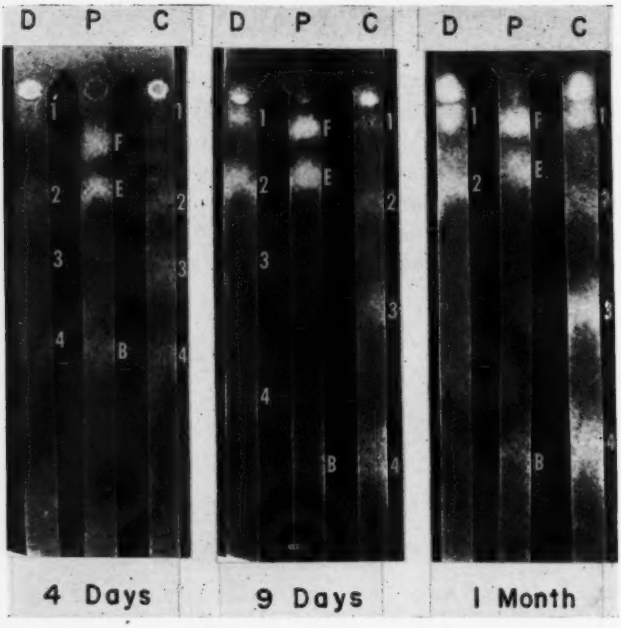

FIG. 4. Chromatograms of the adrenal steroids secreted by sodium deficient rats and pair fed controls.

Each chromatogram consists of three strips labelled D, P, and C. Strip D contains the entire steroid secretion of deficient animals sacrificed on the day indicated and the individual steroids present on the chromatogram are numbered. Strip P is a pilot limb on which hydrocortisone (F), cortisone (E), and corticosterone (B) were placed. Steroid hormones secreted by the adrenals of control animals were placed on Strip C and are also indicated by number.

Previous investigation has shown that steroid 1 is hydrocortisone, steroid 2 is aldosterone, and steroid 4 is corticosterone. The identity of steroid 3 has not been elucidated.

(1) and others (8) who found increased aldosterone excretion in patients on restricted sodium intake.

Although it is well documented that sodium depletion leads to increased production of aldosterone, little attention has been devoted to the effect of this deficiency on the synthesis of other adrenal hormones. It has been stated, however, that the secretion of adrenal steroids other than aldosterone is not altered by sodium restriction (1, 8). In these studies it has been observed that total steroid secretion by the isolated adrenal of normal rats increased progressively as the animals grew. Total adrenal steroid secretion of sodium deficient rats was significantly less than that of controls throughout the period of observation despite growth of the animals and adrenal hypertrophy. The evidence obtained from the chromatograms demon-

strates that the failure of total steroid secretion to increase in sodium deficient rats may be explained by a progressive decline in the production of corticosterone, a hormone which under normal conditions is the major secretory product of the rat adrenal (9).

Since sodium restriction reduces total adrenocortical hormone synthesis, consideration must be given to the effect of this hormonal change on the ability of animals to withstand other types of stress. It seems likely that diminished total adrenal steroid secretion as a result of sodium depletion would make the organism less capable of adapting to other stresses such as cold, heat, infection, and injury. Another fact which must be considered is that aldosterone forms a large proportion of the steroids which are secreted, thus further diminishing the available glucocorticoids. The data obtained in this investigation are not sufficient to substantiate the idea that chronic sodium deficiency may interfere with adaptation of the organism to stress but do suggest that this problem should be further studied.

Aldosterone is normally synthesized by the rat adrenal in small amount and the secretion of other steroids, including corticosterone, is much greater (9). These investigations have shown that, as a result of sodium deficiency, the ratio of aldosterone to corticosterone secretion is changed with larger quantities of aldosterone being produced. It can, therefore, be concluded that the rate of secretion of aldosterone by the rat adrenal can vary independently from that of other hormones. This conclusion is in agreement with that of Singer and Stack-Dunne (9). The results of these experiments also suggest that a specific stress such as sodium depletion may influence synthetic mechanisms concerned with the elaboration of adrenocortical hormones in such a way that the gland secretes increased quantities of the hormone which best enables the organism to withstand this stress. The mechanism by which a specific stress may alter steroid synthesis is not apparent but it seems likely that factors other than ACTH are involved.

SUMMARY

The effects of prolonged sodium depletion on steroid secretion by the isolated rat adrenal have been investigated.

The findings demonstrate that in young, normal animals total adrenal steroid secretion increases with growth.

It was shown that total adrenocortical hormone production by sodium depleted animals was diminished when compared to that of pair-fed controls.

Aldosterone secretion was increased by the adrenals of sodium-deficient rats but production of corticosterone and other steroids was reduced.

It is suggested that a specific stress such as sodium deficiency may influence synthetic pathways concerned with elaboration of adrenocortical hormones through factors other than ACTH.

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ALTERATIONS IN THE ADRENAL CORTEX OF THE RAT INDUCED BY SODIUM DEFICIENCY: CORRELATION OF HISTOLOGIC CHANGES WITH STEROID HORMONE SECRETION[1]

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DEANE, Shaw and Greep (1) demonstrated hypertrophy and alterations in lipid content of the zona glomerulosa of the adrenal cortex in rats fed a sodium-deficient diet. Their observations have been confirmed by other workers (2, 3) and similar changes have been found in human adrenal glands (4). The purpose of the present investigation was to study further the effects of chronic sodium deficiency in order to correlate the histologic appearance with adrenal steroid secretion in the same animals. The experiments were designed so that the early development and progress of the adrenal alterations could be followed. Chemical data, including steroid secretion, are presented in the preceding paper (5). In this paper, histologic results are presented and their significance discussed with respect to adrenal steroid secretion.

MATERIALS AND METHODS

Male rats of the Wistar strain, weighing approximately 60 grams, were fed a sodium-deficient diet *ad libitum* (Table 1). Comparable control rats were fed the same diet to which an adequate amount of sodium chloride was added (Table 1). Food consumption was determined daily and rats in the control group were offered and consumed the same amount of food eaten by the sodium-deficient rats. Animals in both groups drank distilled water *ad libitum*. Equal numbers of animals were selected at random from each group and sacrificed by decapitation 2, 4, 7, 9, 14, 30 and 60 days after the beginning of the experiment. A total of 92 rats was used. At the time each rat was sacrificed, blood was collected for electrolyte determinations and the left adrenal gland was removed and used for steroid determinations as reported in the preceding paper (5).

The right adrenal gland was also removed and fixed in a cobalt-calcium-formalin solution (6) for at least one week. This fixative was found to preserve adrenal lipids better than the usual formalin fixatives. Sections were cut at 5 micra through the center of the fixed adrenal glands with a freezing microtome. They were stained with oil red O for lipid and counterstained with hematoxylin and light green. Frozen sections of a few selected adrenal glands from each group were also stained with Schiff's reagent for aldehydes and by the Schultz reaction for cholesterol. In addition, histologic studies were

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TABLE 1. COMPOSITION OF SODIUM DEFICIENT DIET

Diet		**Salt mixture*	
*Casein	20%	$CaCO_3$	22.600%
		$MgSO_4$	10.000
Sucrose	65	KCl	26.500
		K_2HPO_4	22.500
*Alpha cel	2	$Ca_3(PO_4)_2$	16.600
		$FePO_4 \cdot 2H_2O$	1.620
Corn oil	2	$CuSO_4 \cdot 5H_2O$	0.075
		KF	0.058
*Vitamin mixture	2	$MnSO_4$	0.021
		$CoCl_2 \cdot 6H_2O$	0.010
**Salt mixture	4	KI	0.006
Salt-free butter	5		

*Obtained from Nutritional Biochemicals Corporation.

The vitamin mixture contained the following (triturated in dextrose g.s. 1 kilo.):

Vitamin A concentrate (200,000 U. per gm.)	4.5 grams
Vitamin D concentrate (400,000 U. per gm.)	0.25
Alpha Tocopherol	5.0
Ascorbic acid	45.0
Inositol	5.0
Choline chloride	75.0
Menadione	2.25
P aminobenzoic acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine Hydrochloride	1.0
Calcium Pantothenate	3.0
Biotin	20.0 mg.
Folic acid	90.0
Vitamin B-12	1.35

Control rats received the same diet to which 0.6% NaCl was added.

done on some of the adrenal glands (2 week and 1 month time-intervals) that had been incubated for two hours to determine steroid secretion (5). These were compared with the nonincubated adrenal glands from the same animals.

The total width of the adrenal cortex and the width of the zona glomerulosa were measured with a calibrated ocular micrometer. Amount and distribution of lipid in each of the zones were also recorded. The labels on the slides were masked to assure objectivity on the part of the observer (PMH).

RESULTS

Data demonstrating changes in body weight, adrenal weight, plasma electrolyte concentrations, and adrenal steroid secretion are presented in the preceding paper (5).

The gross and histologic appearance of the adrenal cortex of control rats remained the same throughout the experimental period, except for an increase in total width which occurred as the animals grew (Fig. 1). Characteristically, the zona glomerulosa contained abundant lipid droplets and was demarcated from the deeper cortex by a prominent, lipid-free transition zone. Zona fasciculata also contained lipid, the heaviest deposits occurring in the outer portion. These features are illustrated in Figures 8 to 10.

In contrast, the adrenal cortex of sodium-deficient rats underwent pro-

gressive changes. At autopsy, the glands were grayish to white in color, lacking the pink hue of those from control rats. Microscopically, significant enlargement of the zona glomerulosa was detected as early as the second day. Thereafter, this zone continued to increase in width and became eight times wider than that of control rats after two months of sodium deficiency (Fig. 1). Distribution and physical character of lipid in the zona glomerulosa also changed. *During the first week,* lipid accumulated in greater amounts, but was still stored in the usual droplet form (Figs. 2–4). *After two weeks,* in addition to small droplets, large globules appeared in the outer portion (Fig. 5). *After one month,* almost all the lipid present in the outer one-half of the zona glomerulosa was in the form of globules. In the inner portion of the zone, the cells contained little lipid in contrast to those abutting on the transition zone that were heavily laden with lipid. This lipid, in the innermost cells of the zona glomerulosa, was in the form of small droplets rather than globules (Figs. 6, 15). In some of the rats sacrificed *after two months* of sodium deficiency, large lipid globules were found

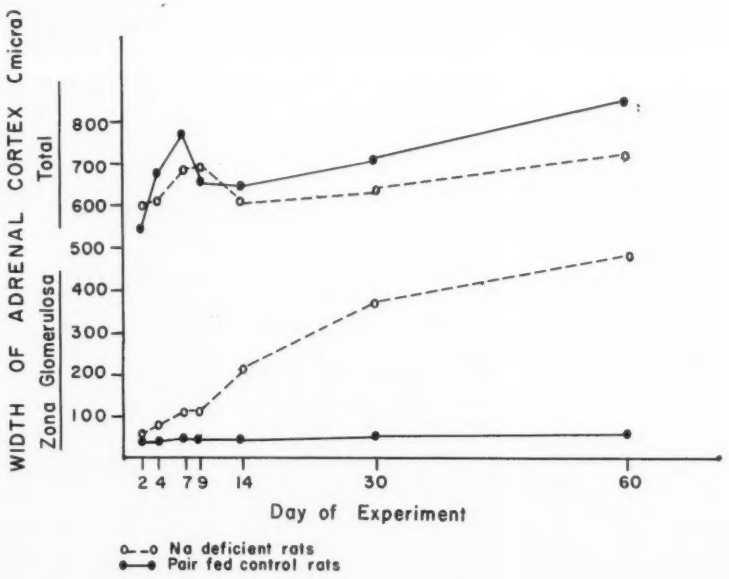
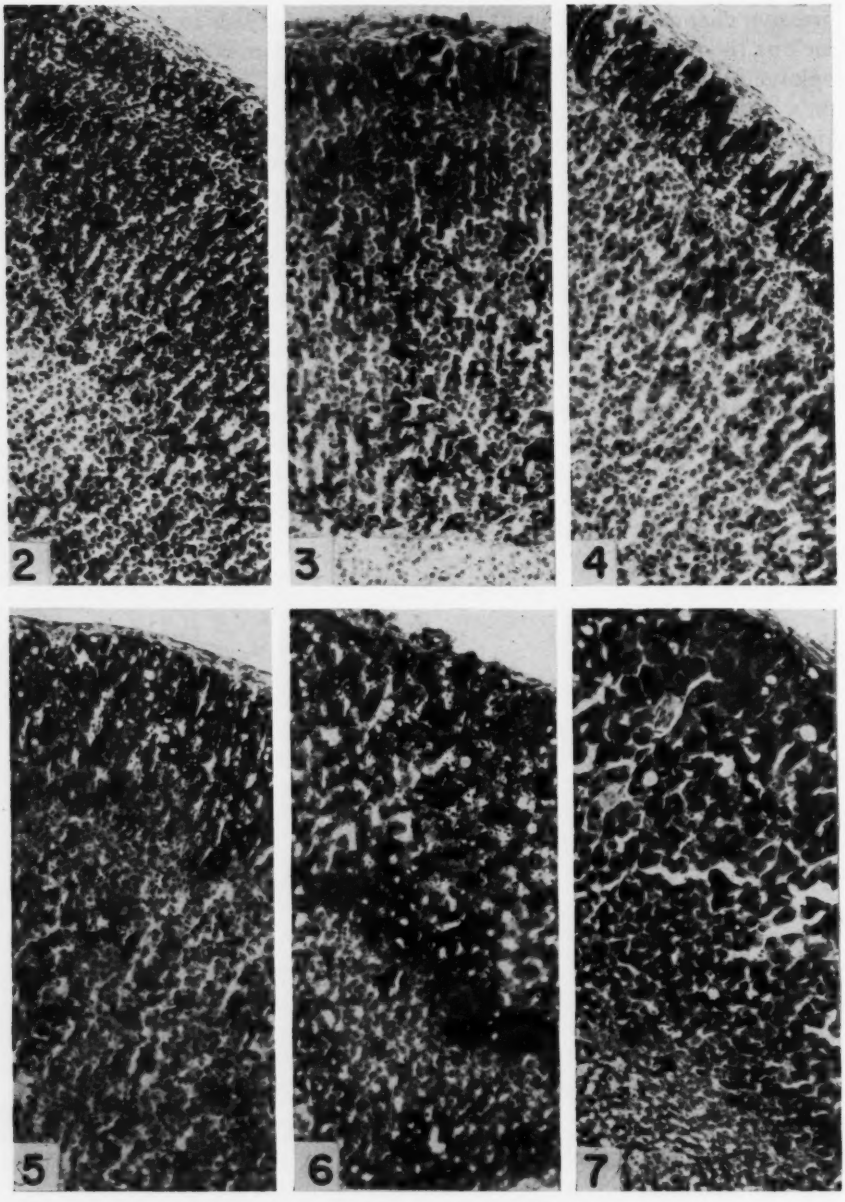


FIG. 1. Graph showing width of the adrenal cortex of sodium-depleted and control rats. Each circle represents a mean value obtained from 5 to 10 rats. Note the rapid increase in width of the zona glomerulosa of sodium-depleted rats (lower broken line). There was only a slight increase in total width (upper broken line). The decrease in width of the remaining cortex (difference between the two broken lines) is accounted for by severe atrophy of zona fasciculata. Significant changes in zona reticularis were not found.



FIGS. 2–7. Frozen sections stained with oil red O, hematoxylin, and light green of adrenal glands from sodium-depleted rats. All illustrations were photographed and printed at the same magnification (130×). The full width of the cortex is included in each figure. Where the medulla is not seen, it lies just out of the field at the lower left. Lipid appears black; nuclei, gray. These photomicrographs, from sections that are typical for the

throughout the entire width of the hypertrophied zona glomerulosa (Fig. 7).

These changes in the zona glomerulosa of sodium-deficient rats were accompanied by striking atrophy of zona fasciculata. After one to two weeks of sodium deficiency, the fasciculata became narrow and depleted of lipid (Figs. 4, 5). By two months, these changes had progressed to such an extent that the zona fasciculata could not be distinguished from the transition zone, which appeared to abut on zona reticularis (Fig. 7).

Enlargement of the zona glomerulosa in sodium-deficient rats, although due in part to an increase in the number of cells, was also due to hypertrophy of individual cells. Furthermore, some of the enlarged glomerulosal cells became distended by the large lipid globules described above, which pushed the nucleus to one side and presented an appearance like that of fat cells (Figs. 11, 12). These globules, like the smaller lipid droplets, gave a positive stain with both Schiff's reagent and the Schultz reaction. In general, the lipid stained by these two reactions, although less in amount and intensity, had the same distribution as that stained by oil red O. This was true of the adrenal cortex in both sodium-deficient and control rats (Figs. 13–16).

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group of rats sacrificed at each time-interval, should be compared with Figures 7, 8 and 9

FIG. 2. Two days of sodium deficiency. The zona glomerulosa is broader than normal but distribution of lipid is not significantly different from that of control rats. Note the prominent lipid-free transition zone between zona glomerulosa and zona fasciculata.

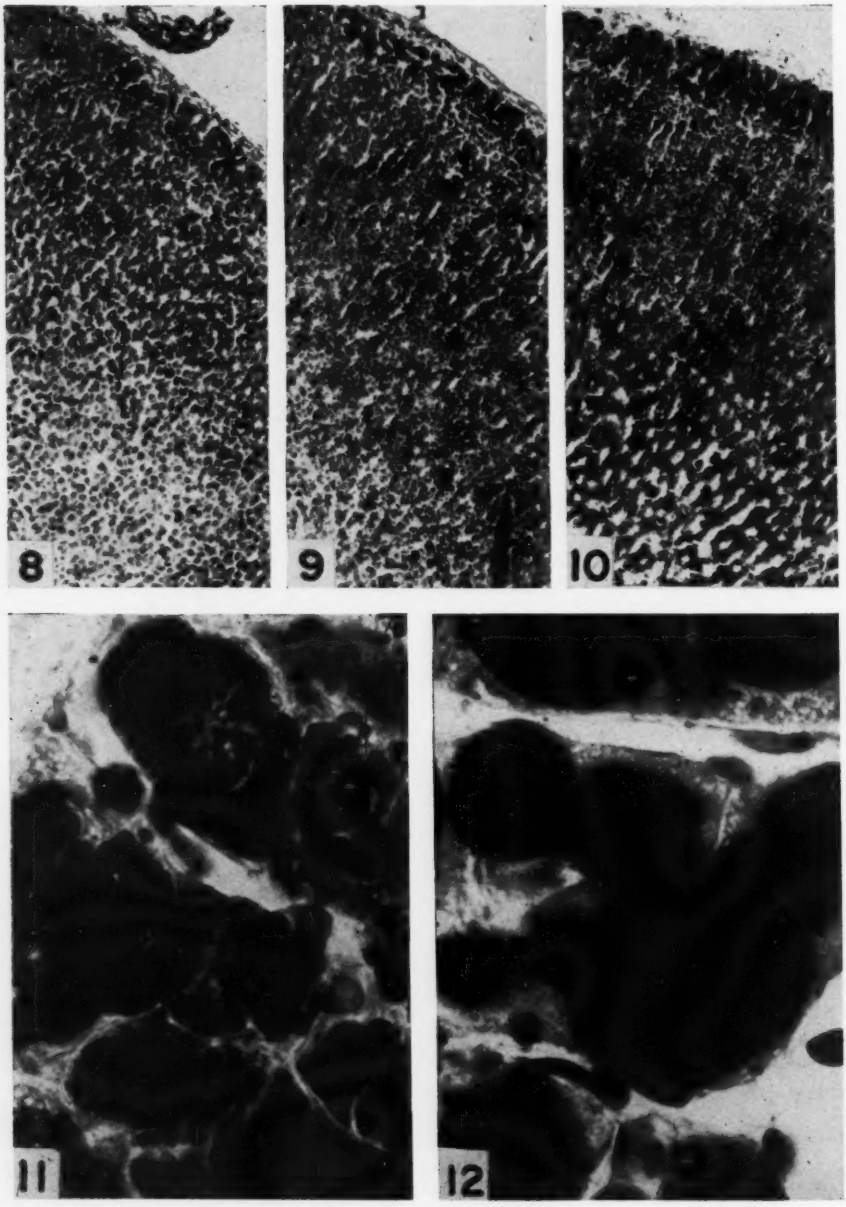
FIG. 3. Four days of sodium deficiency. The zona glomerulosa is wider than in the previous figure. The transition zone is still prominent. There is patchy depletion of lipid in zona fasciculata.

FIG. 4. One week of sodium deficiency. The changes described for Figure 3 are more pronounced. Although the transition zone is no longer prominent due to depletion of lipid in zona fasciculata, it can be identified by its crowded nuclei and absence of lipid.

FIG. 5. Two weeks of sodium deficiency. The zona glomerulosa is very wide and large globules of lipid are present in its outer portion. The clear vacuoles represent lipid globules that have dropped out of the section during cutting and staining procedures. The transition zone is still evident between the glomerulosa and fasciculata. Zona fasciculata, in addition to depletion of lipid, has become decreased in width.

FIG. 6. One month of sodium deficiency. Large globules of lipid in the outer portion of zona glomerulosa are more prominent than in Figure 5. A dense accumulation of lipid in the form of smaller droplets has formed in the inner portion of this zone. Just beneath the glomerulosa is the lipid-free transition zone which is difficult to distinguish from the atrophic fasciculata. Cells of the reticularis, containing scattered lipid droplets, appear at the bottom of the picture and cells of the medulla can be seen at the lower right- and left-hand corners.

FIG. 7. Two months of sodium deficiency. Zona glomerulosa now occupies most of the total adrenal cortex. Large lipid globules extend throughout the width of the zona glomerulosa. Zona fasciculata is absent (this zone was present in some adrenal glands from rats sacrificed at this time-interval but in all cases was severely atrophic). Zona reticularis is present at the lower left-hand corner.



FIGS. 8–10. Frozen sections stained with oil red O, hematoxylin, and light green of adrenal glands from control rats. The magnification of these photomicrographs is the same as that of Figures 2–7 (130×). These figures demonstrate the absence of significant

The histologic appearance of the adrenal gland that had been incubated for steroid studies (5) was essentially the same as that of the nonincubated gland from the same animal. Post-mortem change was minimal. The only changes of note in the incubated adrenal glands were a slight diminution of lipid in the zona fasciculata of control rats and a decrease in width of the cortex, apparently due to collapse of sinusoids. (Figs. 17–20).

DISCUSSION

In 1948, Deane, Shaw and Greep (1) observed hypertrophy and depletion of lipid in the zona glomerulosa of sodium-deficient rats. On the basis of histochemical studies, they interpreted these changes as indicating hyperactivity of glomerulosal cells. They did not find significant alterations in zona fasciculata. In the present investigation, hypertrophy of the zona glomerulosa was also found in sodium-deficient rats, although this enlargement was of greater magnitude than that reported by Deane *et al.* Rather than depletion of lipid, lipid was abundant in the zona glomerulosa of sodium-deficient rats in these experiments, but was stored in a different form, as judged by the appearance of large globules. It is known from the accompanying steroid studies that the zona glomerulosa was secreting at a faster rate in the sodium-deficient rats than in the pair-fed control rats (see below). It follows that production of steroid was also increased, for otherwise the cells would have been depleted of lipid. These observations, therefore, support the conclusion of Deane *et al.*, that the zona glomerulosa becomes hyperactive in sodium deficiency. The differences mentioned above between their findings and those of the present investigation can probably be explained on the basis of more severe conditions in our experiments. The rats were younger, in a period of rapid growth, and therefore more susceptible to a deficiency such as this one, and they drank distilled water rather than tap water.

In addition to changes in the zona glomerulosa, severe atrophy of zona fasciculata occurred as a result of sodium deficiency in the present investigation. It was of value that both steroid secretion (see preceding paper (5))

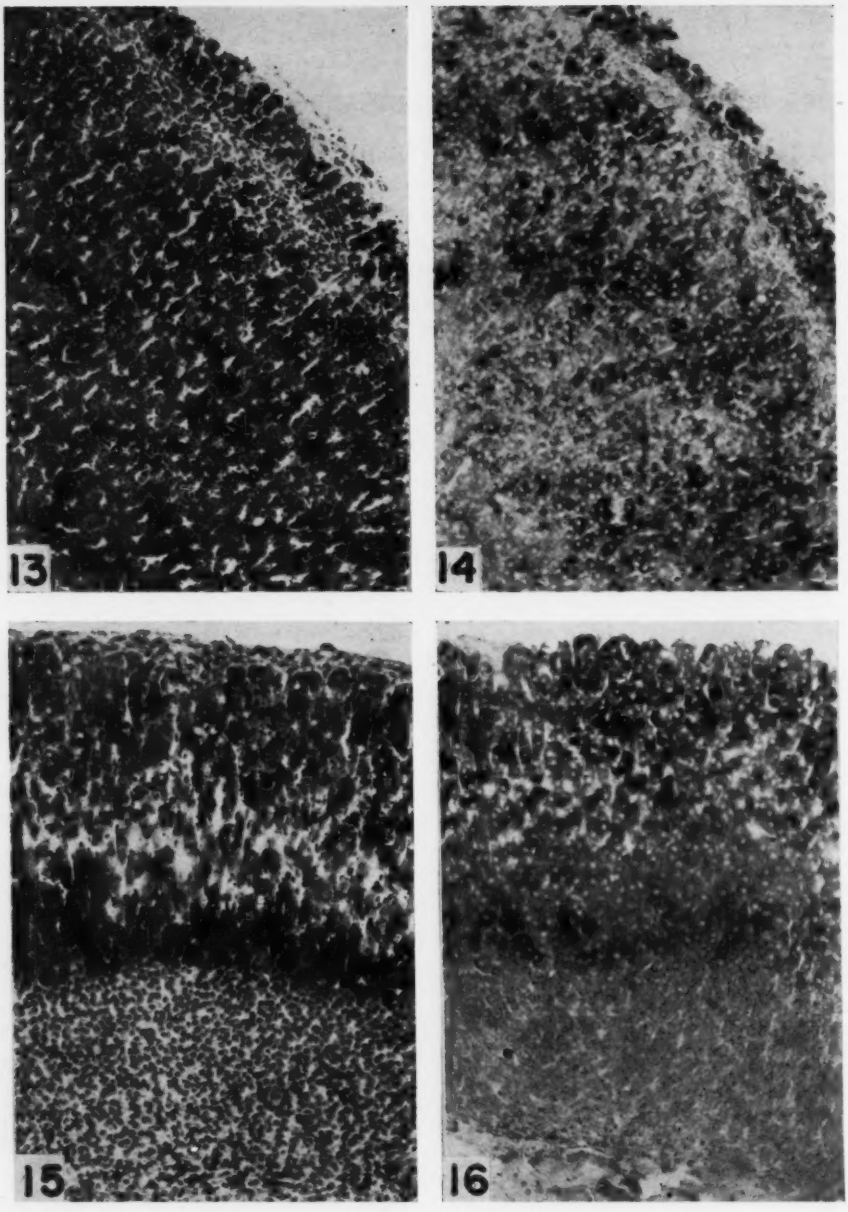
changes in the adrenal cortex of control rats throughout the experimental period of two months. They should be compared with Figures 2–7.

FIG. 8. Two days; Fig. 9. Two weeks; Fig. 10. Two months.

FIGS. 11 AND 12. Oil immersion photomicrographs, both at the same magnification, illustrating the form of lipid storage in the zona glomerulosa of the adrenal cortex. Frozen sections stained as above. Lipid is black; nuclei, gray; 1200×.

FIG. 11. From a control rat (2 months) showing lipid in the form of small droplets. Nuclei are located in the center of the cells.

FIG. 12. From a sodium-deficient rat (2 months). Large lipid globules have distended glomerulosal cells and pushed the nuclei to one side. (Note flattened nucleus with a prominent nucleolus just to the left of the large lipid globule near the center of the picture).



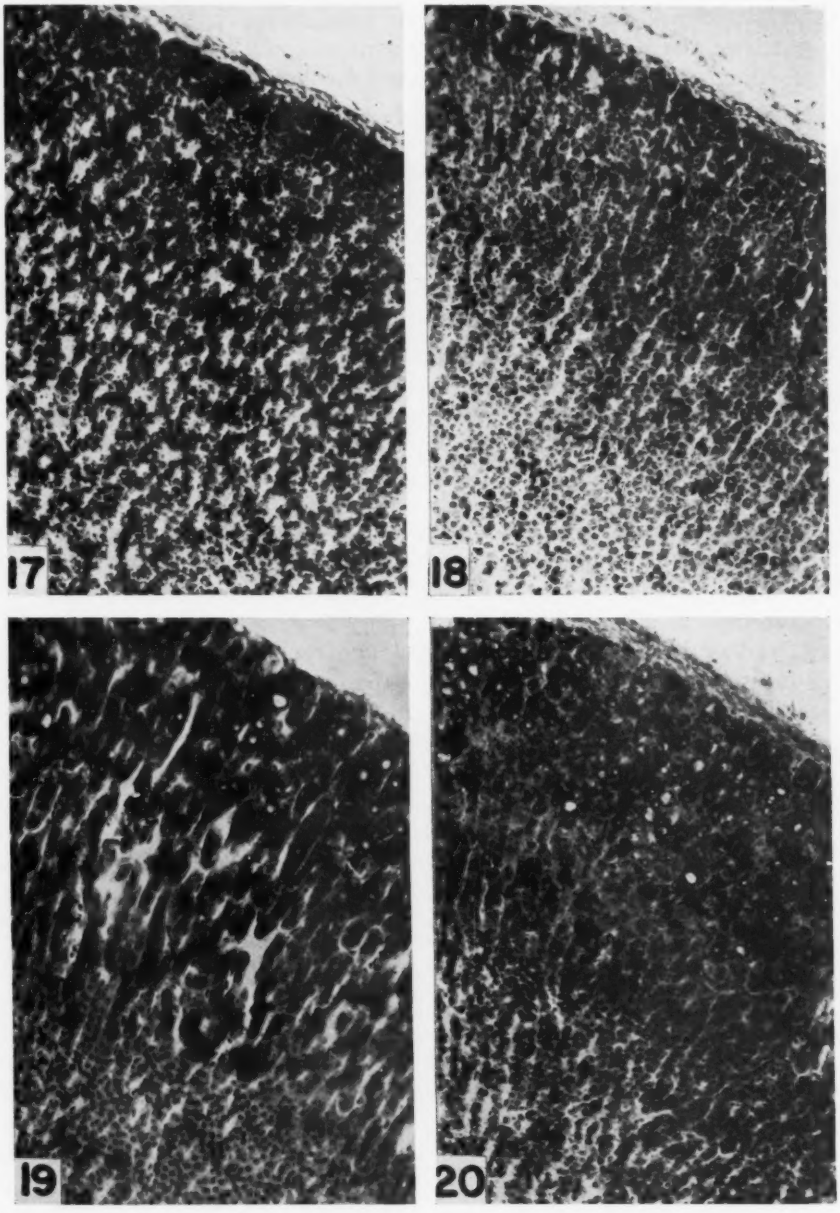
FIGS. 13–16. Frozen sections of the adrenal cortex, all photographed at the same magnification (140×).

FIG. 13. From a control rat. Section stained with oil red O, hematoxylin, and light green. Note distribution of lipid (black).

FIG. 14. Frozen section from the same adrenal as in Figure 13 but stained with Schiff's reagent. Although somewhat less in amount, the reactive lipid has the same distribution as the lipid stained with oil red O.

FIG. 15. From a sodium-deficient rat (one month). Frozen section stained with oil red O, hematoxylin, and light green. The hypertrophied zona glomerulosa occupies the upper half of the illustration. Note complete absence of lipid in the fasciculata.

FIG. 16. Frozen section from the same adrenal as in Figure 15 but stained with Schiff's reagent. Note lack of deeply staining lipid in zona fasciculata. The large lipid globules in the outer portion of zona glomerulosa are positive, as is the finely distributed lipid in its inner portion.



FIGS. 17–20. Frozen sections stained with oil red O, hematoxylin and light green. All photographs are at the same magnification (140×).

FIG. 17. From a control rat. The adrenal gland had been removed at the time the

(*Explanation of figures continued on next page*)

and histology of the adrenal cortex had been studied in the same animals. Otherwise, the fact that both the glomerulosa and fasciculata were altered by sodium deficiency would have been difficult to explain on the basis of previous concepts. As mentioned above, Deane *et al.* (1) had demonstrated hypertrophy of the zona glomerulosa but had not found changes in the fasciculata in sodium deficiency. This observation, together with the fact that only the fasciculata is altered following hypophysectomy or with injections of ACTH (7), was used as evidence for the conclusion that electrolyte-regulating hormones are produced by zona glomerulosa and glucocorticoids by zona fasciculata. Because of the dual nature of the present investigation, it was possible to demonstrate not only that increased aldosterone secretion accompanied hypertrophy of zona glomerulosa but also that depression of glucocorticoid secretion, particularly corticosterone (5), was associated with atrophy of zona fasciculata. Therefore, these observations, based on more direct methods, support the concept proposed by Deane and associates (above). That aldosterone is elaborated specifically by zona glomerulosa has also been demonstrated recently by others, including Giroud *et al.* (8) who used a different approach. These workers found that only the "capsule" (including glomerulosa and a small part of fasciculata) of decapsulated adrenal glands would secrete significant amounts of aldosterone *in vitro*.

The underlying reason or mechanism for atrophy of zona fasciculata and depressed glucocorticoid secretion in these sodium-deficient rats is not readily apparent. Obviously, it was not due to non-specific stress which would have caused enlargement of this zone. Since the level of sodium in the diet was the only known variable, it seems likely that atrophy of zona fasciculata was secondary. Indeed, the rapidly enlarging zona glomerulosa, confined by a connective tissue capsule, might well have produced enough pressure on underlying cells to induce "compression atrophy" of zona fasciculata. Such a mechanism would explain why other workers had not

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animal was sacrificed and placed in fixative. This figure should be compared with Figure 18.

FIG. 18. The opposite adrenal from the same rat as in Figure 17. This adrenal gland had been incubated for two hours for steroid studies (see preceding paper (5)) before being placed in fixative. The zona glomerulosa appears the same but lipid in the fasciculata is not as abundant as in the nonincubated gland. Note that the sinusoids are less prominent than in Figure 17.

FIG. 19. From a sodium-deficient rat (one month). Details the same as for Figure 17. Note the prominent sinusoids, especially in the inner portion of zona glomerulosa.

FIG. 20. The opposite adrenal gland from the same animal as in Figure 19. This adrenal had been incubated for two hours for steroid studies. Sinusoids are collapsed (compare with Fig. 19), causing shrinkage of zona glomerulosa. Lipid distribution in zona glomerulosa is the same as in the non-incubated gland. The clear vacuoles, also present in the previous illustration, are artefacts caused by lipid globules that have dropped out (note dark rings of adherent lipid lining the vacuoles).

observed this effect, because the rate and degree of hypertrophy of zona glomerulosa was much greater in our experiments.

It is concluded, therefore, that when sodium depletion is sufficiently severe, the zona glomerulosa hypertrophies and produces more aldosterone at the expense of the fasciculata and its glucocorticoids. These experiments have provided further evidence that the different zones and functions of the adrenal cortex can vary independently of each other. It can only be concluded that other factors, in addition to ACTH, must be involved in regulating adrenocortical growth and function.

SUMMARY

Following sodium depletion in rats, extreme hypertrophy and altered lipid storage, indicating hyperactivity, were observed in the zona glomerulosa of the adrenal cortex. These changes were progressive, first detectable after two days and thereafter increasing in degree throughout the two-month period of observation. Although alterations in the zona glomerulosa were more severe, they confirmed findings of previous workers. In addition, atrophy of the zona fasciculata was found in the sodium-depleted rats. The probable reasons and mechanism responsible for this unexpected finding are suggested.

Histologic results described in this paper are interpreted on the basis of steroid secretion by adrenal glands from the same animals, as presented in the preceding paper (5). The value of dual studies of this nature is emphasized. In adrenal glands from the same rats, increased aldosterone secretion was associated with hypertrophy of the zona glomerulosa and depressed glucocorticoid secretion with atrophy of zona fasciculata. These findings present further evidence for functional specificity of the zones in the adrenal cortex.

Acknowledgments

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OXYTOCIN IN HUMAN FEMALE BLOOD

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A METHOD for the extraction of oxytocin from rat blood has been described by Bisset & Walker (1). They found in blood taken from rats under ether anaesthesia a range of 1.7 to 6.8 mU. oxytocin/ml. with a mean of 3.9 mU./ml. Using a slight modification of their method of extraction, we have determined the oxytocic activity of bloods taken from non-pregnant females; healthy pregnant females towards the end of pregnancy; and from women in the first stage of labour.

METHOD

Preparation of the extracts.—Samples of venous blood were collected from the various subjects and prevented from clotting by adding heparin. Each sample was mixed separately with 9 vol. 80% (V/V) ethanol, and N-H_2SO_4 was added until the solution was blue to Congo Red. This was allowed to stand for 3 hours with frequent shaking. The precipitate was removed by centrifuging and the supernatant concentrated to about one-tenth its original volume by passing warmed air over the liquid in a shallow dish. The temperature of the extract was kept below 45° C. The concentrated extract was again centrifuged and the supernatant retained. This, together with the supernatant obtained on centrifuging the 80% (V/V) ethanol washings of the dish, was evaporated almost to dryness in air below 45° C. The residue was then taken up in 10 ml. distilled water and centrifuged, and the supernatant occasionally evaporated to dryness as above but more usually left to evaporate overnight at room temperature in an open dish. The new residue was then dissolved in up to 5 ml. distilled water and neutralised by the addition of N and N/10 sodium hydroxide. If the solution was left for any length of time before the assay, the extract was made acid to ensure its stability.

Method of assay.—Extracts were assayed for oxytocic activity (O.A.) against Pitocin (Parke, Davis & Co.) on the isolated uterus of virgin Wistar strain rats in oestrus following the method described by Bisset and Walker (1) (1954), except that the bath volume was 10 ml. and the doses of Pitocin standard used were 0.04–0.2 mU./ml. Thioglycollic acid was adjusted to pH 7.5 with $NaHCO_3$, added immediately to part of some of the extracts to make a final concentration of 0.01 M at pH 7.5 and left for 30 min. The O.A. (oxytocic activity) of the extracts that was destroyed (2) has been considered as oxytocin.

In some extracts, after thioglycollate treatment, there was residual activity which could have been due to potassium or to 5-hydroxytryptamine (HT). When these were present as interfering substances, they were removed from the extract by dialysis for 30 min. through a cellophane membrane against running distilled water and when necessary dibenamine hydrochloride (5 μg./ml.) was added to the bath so as to block the action of 5-hydroxytryptamine.

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RESULTS

(a) *Preliminary investigation*

The potassium concentration in a number of extracts was estimated directly by a flame photometer, and the amounts of this ion contained in the largest doses of extract used were compared with the uterine threshold for K. The highest value for potassium was 0.015 mEq. from an undialysed sample, whilst the lowest threshold dose was 0.1 mEq.

Frequently after treatment with thioglycollate, the residual activity of the extract persisted after eliminating potassium or 5-hydroxytryptamine. Yet this activity was removed by incubating the extracts with plasma collected from a normal pregnant woman. This plasma has been shown to contain an enzyme which destroys standard oxytocin (3). Since all O.A. disappeared after treatment with plasma, K was not likely to be an interfering substance. To eliminate the possibility that some substances in the extracts might protect oxytocin from inactivation by thioglycollate, Pitocin was added to extracts of blood taken during pregnancy and labour. The oxytocin was completely inactivated by sodium thioglycollate.

In the initial estimations a cellophane membrane was used. It allowed K and HT to dialyse through but retained oxytocin. Further supplies of this were unobtainable so another cellophane membrane, which was partly permeable to oxytocin was used. In order to correct for this leakage, a control oxytocin dialysis was run when the extracts were dialysed. If the extracts were not assayed immediately, they were acidified (pH 2–3) and stored in the refrigerator. In spite of these precautions there was some (20–40%) loss of oxytocic activity in 24 hours.

Concentrated solutions of Pitocin (1 U./ml.) lost no activity when stored in the deep freeze or refrigerator for 3 weeks. Dilute solutions (10 mU./ml.) however, behaved differently, for in an acid medium, refrigerated for 2 days, 44% of the Pitocin was lost and, furthermore, in an alkaline or neutral solution there was a loss of >85%. Pitocin is most stable in acid medium. (We were surprised to observe that dilute solutions of Pitocin lost <10% of their activity if first boiled before storing at 4° C for 5 days at pH 7.4.)

In recovery experiments where 50 mU. of standard oxytocin (Pitocin) were added to 12 ml. venous blood and then extracted, (but not dialysed) the recoveries were in the order of 59% (blood taken during labour); 50% (nonpregnant); and 78% (blood taken during pregnancy).

(b) *Oxytocic activity of the extracts*

Table 1 shows that all the extracts tested possessed oxytocic activity. After eliminating the possible effects of K and HT, this activity appeared to be due to both oxytocin and a second unidentified oxytocic substance. The former but not the latter was unstable in thioglycollate, yet both were

TABLE 1. OXYTOCIC ACTIVITY OF ACID-ALCOHOL EXTRACTS OF BLOOD FROM NON-PREGNANT AND PREGNANT WOMEN AND DURING PARTURITION

Type	Non-dialysed mU./ml.				Dialysed mU./ml.				Pitocin recovered during dialysis
	Thioglycollate		Oxytocin	% oxytocin total activity	Thioglycollate		Oxytocin*	% oxytocin Total activity	%
	Before	After			Before	After			
Non-pregnant									
1					1.90	0.80	1.10	57.9	
2					2.75	1.48	1.27	46.2	
3					2.65	1.90	0.70	26.4	
4					<3.2				
5					>8.80	<1.00	>7.8	>88.6	100
6					1.45	<0.40	1.05 1.45	72.2 100.0	
7					0.81	0.41	0.40	49.4	
8					1.10	0.60	0.50	45.4	
9	2.20	1.26	0.90	40.9					
10	2.10	1.20	0.90	42.8	0.82	>0.30	0.52 0.82	62.3 100.0	50
11	1.02	>0.80	<0.20	<19.6					
12	0.45								
13	0.40								
14					<1.20				
15					6.40	2.90	3.50	54.7	
16					1.20	>1.00	<0.20	<16.6	
17					2.70	<0.50	2.20 2.70	81.2 100.0	53
18					1.20	1.30	0	0	
19					0.88				60
Pregnant									
1					3.10	0.56	2.50	80.6	
2					3.10	0	3.10	100.0	
3					1.40	0.45	0.95	67.8	
4					1.30	<0.20	>1.10	>84.6	
5					2.30	0	2.30	100.0	50
6					8.00	0	8.00	100.0	
7					5.30	0	5.30	100.0	
8					43.00	0	43.00	100.0	
9					20.00	0	20.00	100.0	
Labour									
1					1.40	0.80	0.60	42.90	
2					0.62	<0.47	>0.15	>24.20	60
3					0.70	<0.42	>0.3	>42.90	
4					0.90	<0.55	<0.35	<38.8	
5	0.39	0.39	0	0					
6	0.44	0.44	0	0					
7	0.51	0.51	0	0					
8					0.71	<0.33	>0.38	>53.50	
9					1.70	0.53	1.17	68.80	30
10					0.45	0.34	0.11	24.40	
11					0.37	0.27	0.10	27.00	

* The oxytocin figures are uncorrected for loss through dialysis membrane.

destroyed by an enzyme in pregnancy plasma. Extracts from pregnancy blood had the highest O.A., whilst extracts of blood from healthy nonpregnant women and from women during labour (1st stage, cervix 3 finger dilated) were of the same order of activity. In pregnancy, of 9 samples treated with thioglycollate the activity in 6 could be attributed entirely to oxytocin, and this hormone was responsible for >68% of the O.A. in the remaining 3. In both nonpregnancy and labour, however, the oxytocic activity of the extracts ascribed to oxytocin showed a wider scatter.

DISCUSSION

Thioglycollate is assumed (4) to partition the oxytocic activity of the extracts into oxytocin and a second oxytocic substance. Bisset and Walker (1) also noted residual oxytocic activity in extracts of rat's blood after

thioglycollate treatment. We have measured this thioglycollate resistant oxytocic activity against standard Pitocin. The slopes of the regression lines of oxytocin and the second substance are not exactly parallel, but the difference of their slopes is no larger than might reasonably have been expected (Fig. 1). We investigated this residual activity because it frequently accounted for a large percentage of the total oxytocic activity, and because of the intrinsic interest in an active substance which was non-dialysable.

When all the activity was removed by thioglycollate, it was assumed that this loss was due to the destruction of oxytocin by thioglycollate, and when there was no loss of activity after thioglycollate, it was concluded that no oxytocin was present. However, the results did not always fall into these two categories, for instance, in the second nonpregnant subject, 46% of the total activity was estimated to be due to oxytocin in the mixture.

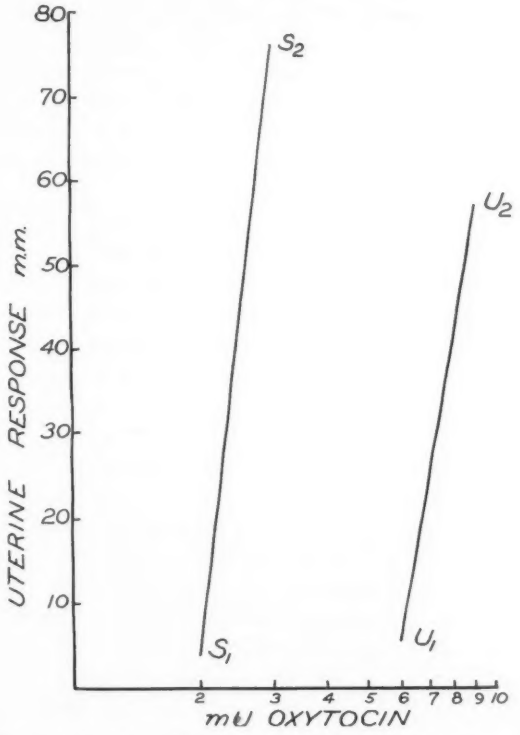

FIG. 1. Abscissa: log dose in milli-units of oxytocin (Pitocin).

Ordinate: uterine response in mm.

S_1 and S_2 are the responses for low and high doses of standard

U_1 and U_2 are the responses for the low and high doses of unknown

Validity test	*Index*	*Variance*	*S.D.*	*t*	*P*
Parallelism	G = −23.5	V = 220	14.8	1.6	0.5–0.1

In spite of the difficulty of interpretation of the results, we tried to estimate the fraction of the total activity in each group that might be due to oxytocin (see Table 1). In the nonpregnant female and during labour, the proportion of total activity attributed to oxytocin varied widely. Extracts of blood collected from healthy pregnant women showed the highest per cent of oxytocin to total activity, and in 6 of the 9 cases investigated, all the O.A. was considered to be due to oxytocin. The total O.A. of the extracts was higher during normal pregnancy than during labour; in fact it was invariably slightly higher in nonpregnant females than in women during labour. It seems extraordinary that, in the last few weeks of pregnancy when the uterus is relatively quiescent, the O.A. of the blood is at its peak, whereas during labour (and in the nonpregnant) when the uterus is actively contracting, the O.A. is low. Furthermore, in pregnancy the oxytocic activity is almost entirely due to oxytocin whereas in labour (and nonpregnancy) it is due to a mixture of the two oxytocic substances. The second oxytocic substance may have a role in labour, either by initiating uterine movements or by sensitising the uterus to oxytocin. These results, however, give no information on the sensitivity of the human uterus to the two oxytocic substances of the blood during labour, and so neither support or negate the frequently cited contention that during parturition, the uterus becomes sensitised to endogenous oxytocin.

In the human (5), during suckling, most of the oxytocic activity of the blood extracts results from the second oxytocic substance which is postulated to be of hypothalamic-posterior pituitary origin.[1] It is just possible that this region elaborates 2 oxytocins, and that there is a simultaneous release of them in variable ratios during nonpregnancy, pregnancy, labour and lactation.

SUMMARY

Using a modification of the method of Bisset and Walker 2 oxytocic substances have been extracted from blood taken from healthy nonpregnant adult females, and from women during normal pregnancy and in the first stage of labour. The possibility that K and HT in the extracts might be interfering substances in the assay procedure was removed by dialysis or by adding dibenamine hydrochloride to the organ bath.The two oxytocic substances were assayed on the isolated sensitised rat uterus, and were differentiated from one another by treatment with sodium thioglycollate, which inactivates oxytocin but not the second oxytocic substance.

The ratio of oxytocin to the second substance was high in pregnancy, and extremely variable in labour and in nonpregnancy; the total oxytocic activity was highest in pregnancy.

[1] Both oxytocin and the second unidentified oxytocic substance have recently been extracted by us from the hypothalami of cats, cows and rats.

The site of production of the second oxytocic substance is possibly in the hypothalamus-posterior pituitary region.

Acknowledgment

We wish to thank Professor W. V. Macfarlane for his interest in this work; Miss Vivienne Roberts for her technical assistance; and the National Health and Medical Research Council (Australia) for a grant.

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HIGH POTENCY OF 2-METHYL-9α-FLUOROHYDROCORTISONE FOR LIFE MAINTENANCE AND REVIVAL FROM INSUFFICIENCY OF ADRENALECTOMIZED DOGS[1]

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TWO new synthetic analogs of hydrocortisone: 2-methylhydrocortisone (2-methyl F), and 2-methyl-9α-fluorohydrocortisone (2-methyl FF), have become available (1) and early reports have called attention to the high Na-retaining activity of 2-methyl FF (2–4). This steroid has been stated to be 49 times more active in this respect than DCA and 155 times more potent in inducing K excretion in acute assays on adrenalectomized dogs (2). Investigators employing rats as test objects observed that the Na-retaining activity of methylated FF is 90 times that of DCA (4). The present writers have found the FF compound to be the most potent mineralocorticoid known both for maintaining adrenalectomized dogs symptom-free and for its remarkable activity in reviving such animals from severe insufficiency in record time with restoration of normal activity, vigor and serum electrolyte pattern.

METHODS

Long term adrenalectomized dogs, routinely maintained on DCA, were used for assay and maintained at each dose level for 10 days. At the close of these periods arterial pressure and blood samples were taken and the daily dose reduced by 50%. The assay was terminated when the dose eventually reached the level where either frank symptoms of insufficiency appeared or certain of the blood constituents indicated prodromal signs of impending adrenal failure. The dogs were then given i.v. injections of 2-methyl FF which quickly restored them to normal health. The diet was rigidly controlled, the total amount of Na and K ingested daily being 1.47 gm. Na and 0.94 gm. of K. The free alcohol of the steroid was prepared for injection by dissolving it in absolute ethanol and diluting it drop-wise to 10% with warm distilled water immediately before using. Injections were administered subcutaneously twice daily at 9 a.m. and 5 p.m.

RESULTS

The minimum maintenance dose (MMD) level as referred to hereafter is the daily amount of steroid necessary to maintain the dog free from

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symptoms and the arterial pressure and blood constituents little, if any, changed from their initial or control values (5).

2-methylhydrocortisone. Two dogs were tested on the compound, but since the results obtained were practically identical for both animals, it was not considered worthwhile to add to the number of assays. The pertinent data are recorded in Table 1. This steroid is somewhat less potent for maintaining health than DOC insofar as the type of test used in these experiments is concerned. For example, the two dogs receiving the compound (Dogs 4, 5. Table 1) required 500 μg./dog/day in order to maintain arterial pressure and blood constituents at normal levels. Lowering the dose to 250 μg./day led to mild increases in blood urea nitrogen, hemoconcentration and serum K in both animals and a decline in arterial pressure in Dog 5. Therefore, the MMD was established at the 500 μg. level. Symptoms of insufficiency did not become apparent for 20 days after the MMD had been reached. Earlier assays on DOC showed that MMD of this compound is approximately 250 μg./dog/day (6).

2-methyl FF. Three animals were used for assaying this steroid. The

TABLE 1. ADRENALECTOMIZED DOGS MAINTAINED ON 2-METHYL FF AND 2-METHYL F

Dog	Dose/ dog/day	Exper. period	Wt.	B.P.	Blood urea N.	Hb.	Hmct.	R.B.C.	Blood sugar	Serum Na	Serum Cl	Serum K
	micro-grams	days	Kg.	mm. Hg	mg.%	gm.%	%	10^6/ cu. mm.	mg.%	meq./ L.	meq./ L.	meq./ L.
						2-methyl FF						
		Initial	17.5	110	24.5	14.0	39.8	6.6	79	141.8	110.7	5.8
	25.0	10	18.2	108	15.0	12.4	27.1	4.4	85	154.2	119.6	3.3
	12.5	10	17.9	112	14.5	11.4	32.0	4.2	89	152.5	120.3	3.9
	6.25	10	18.1	128	20.2	13.4	35.0	5.2	80	154.0	115.5	4.1
1	3.125	10	18.4	106	22.0	13.6	35.1	6.1	85	147.5	115.9	4.6
	1.56	10	17.6	102	30.8	15.0	38.0	5.8	85	145.0	111.4	5.4
	0.781	10	17.0	92	38.5	15.0	39.4	7.3	86	140.0	109.0	7.3
	0.39	3	16.8	79	47.0	15.7	—	—	90	138.0	105.6	7.3
		Initial	14.2	102	24.8	13.6	37.2	5.2	73.5	141.3	110.0	5.3
	25.0	10	14.6	108	17.7	11.5	26.9	4.8	83.0	156.3	121.7	3.3
	12.5	10	14.8	116	13.4	11.1	28.6	4.9	82.5	155.0	118.2	2.9
	6.25	10	14.8	126	13.5	12.6	32.6	4.8	75.0	156.0	120.3	3.8
2	3.125	10	15.0	120	18.5	13.7	34.7	5.1	73.0	155.5	116.3	4.0
	1.56	10	14.5	124	26.3	13.8	37.0	5.8	81.0	147.5	116.4	4.7
	0.781	10	14.0	96	31.5	15.7	37.1	5.8	85.0	144.0	109·0	5.1
	0.39	10	13.7	84	62.5	17.4	39.7	6.2	90.0	126.0	103.0	8.0
		Initial	13.4	110	19.0	12.2	37.5	4.2	76.5	143.5	113.5	5.2
	12.50	10	13.4	136	11.8	11.7	33.5	4.5	75.5	141.5	110.0	3.5
	6.25	10	13.4	128	13.6	12.6	36.6	5.7	74.0	144.0	114.0	3.2
	3.125	10	13.4	126	12.0	15.0	36.3	5.6	75.0	139.0	115.5	3.6
3	1.56	10	13.4	126	12.3	15.0	33.2	4.7	70.0	139.0	114.5	3.7
	0.781	10	13.3	106	12.5	15.1	33.2	5.2	70.0	135.0	113.5	4.3
	0.39	7	13.0	72	45.0	15.0	42.0	7.0	78.0	128.5	102.0	7.0
						2-methyl F						
		Initial	12.3	118	18.0	14.3	32.6	5.5	77.5	146.0	116.0	4.4
	500	10	12.8	122	17.2	12.2	35.6	5.8	90.0	151.0	117.0	5.0
4	250	10	12.6	112	40.0	16.1	44.2	6.9	86.5	148.0	114.0	6.6
	125	10	12.4	95	80.0	15.9	43.6	6.6	105.0	132.0	103.0	7.6
		Initial	11.4	119	18.7	12.2	39.2	6.8	72.5	146.0	112.1	5.2
	500	10	11.5	110	18.4	13.7	39.5	6.4	85.0	146.0	113.5	5.7
5	250	10	11.4	90	21.0	15.7	41.0	7.2	80.0	146.0	113.5	6.3
	125	10	11.3	88	35.0	17.3	40.5	6.8	85.0	134.5	109.3	7.4

Doses underlined are minimum maintenance levels.

starting dose was 25 μg./dog/day for Dogs 1 and 2, and 12.5 μg./day for Dog. 3. These doses proved to be excessive and caused the assay to extend over 60–70 days before marked signs of adrenal failure became evident. The MMD of 2-methyl FF was 1.56 μg./day for Dogs 1 and 2 and between 0.781 and 1.56 μg./day for Dog 3. Several of the animals that were used in the earlier assays of aldosterone, the 9 alpha halo compounds and other steroids, were also employed in the present experiments (5–7).

The failing dose; *i.e.*, the quantity of hormone the animals were receiving when gross symptoms appeared, was 0.39 μg./dog/day. The mean arterial pressure exhibited a modest though sustained rise during those periods when the dosage was highest, but when this was decreased the blood pressure slowly declined (Table 1). The serum Na rose sharply in Dogs 1 and 2; *i.e.*, from 141.8 to 154 and from 141.3 to 156 meq./L. This electrolyte did not decline to levels characteristic of adrenal insufficiency until the daily dose of 2-methyl FF was reduced to 0.39 meq./L. Likewise the serum K failed to increase significantly until very low dose levels were reached. Eventually the three animals exhibited serum K concentrations of 7–8 meq./L. coincident with the appearance of muscular weakness. It will be noted (Table 1) that serum K was depressed below the initial value at the higher steroid dosage; presumably this was due to increased renal elimination of the cation.

EFFECTIVENESS OF 2-METHYL FF FOR REVIVING DOGS FROM SEVERE ADRENAL INSUFFICIENCY

Six dogs were used in the experiments, although the data from but five are summarized in Table 2. The daily maintenance dose of DCA was withheld 7–13 days, or until the dogs presented gross signs of adrenal insufficiency. The designation "Day Bled," Column 2 in the table, refers to the day DCA injections were stopped and the initial blood samples taken. The next date listed in the same column is the day restorative measures were instituted and also indicates the interval elapsing between cessation of maintenance therapy and the onset of severe symptoms. The total dosage administered to dogs 1–4 was 250 μg. given in 2 doses i.v., 150 μg. the first and 100 μg. the second twenty-four hours.

The rapid return of activity and vigor was dramatic considering the severity of the symptoms displayed. Dogs 1–3 were in collapse and unable to rise or to stand erect when placed on their feet. The serum K was greatly elevated and profound muscular asthenia was associated with cardiac disturbances. One animal, Dog 4, could walk but exhibited marked weakness and spasticity. However, within 4–6 hours after injection with 2-methyl FF, the dogs were jumping about in their cages eager for food. They ate full rations and were free of all signs and symptoms of insufficiency. The striking changes in arterial pressure and blood constituents accompanying restoration of activity and vigor in these dogs are shown in Table 2. Serum

TABLE 2. REVIVAL OF DOGS FROM ACUTE ADRENAL INSUFFICIENCY WITH 2-METHYL FF

Dog	Day bled	Dose/ dog/ day	B.P.	Blood unea N.	Hb.	Hmct.	R.B.C.	Blood sugar	Serum Na	Serum Cl	Serum K	Condition of animal
		micro-grams	mm. Hg	mg.%	gm.%	%	10^6/ cu. mm.	mg.%	meq./ L.	meq./ L.	meq./ L.	
1	1	0	106	20.6	14.5	32.8	6.39	81.5	145.5	114.1	5.7	Normal
	13	150	68	86.0	14.5	44.2	6.70	83.5	129.0	110.0	9.6	Collapse
	14	100	112	27.5	12.0	35.3	6.20	77.5	—	—	—	Active
	15	0	118	14.5	9.7	27.7	5.20	75.0	141.0	114.0	3.8	Normal
2	1	0	116	21.8	12.6	34.4	5.47	80.0	148.0	116.0	4.6	Normal
	7	150	54	92.0	16.9	43.1	6.92	71.0	128.0	100.0	9.2	Collapse
	8	100	104	25.0	13.4	32.8	5.95	86.0	—	—	—	Active
	9	0	129	13.0	11.4	28.7	4.16	88.5	140.0	110.9	3.6	Normal
3	1	0	120	24.0	13.1	37.0	6.14	78.0	145.0	112.1	5.5	Normal
	9	150	72	80.0	14.5	38.4	6.11	77.0	127.5	109.0	9.5	Prostration
	10	100	112	23.0	10.2	29.1	4.9	85.0	133.0	108.6	3.6	Active
	11	0	118	13.5	9.4	29.0	4.7	80.5	138.0	107.7	3.5	Normal
4	1	0	114	28.0	13.8	32.8	6.3	84.0	146.0	114.0	4.5	Normal
	13	150	68	129.0	13.6	34.2	6.5	75.0	125.0	99.8	8.5	Symptoms
	14	100	118	30.0	10.5	27.4	4.8	90.0	—	—	—	Active
	15	0	116	15.6	9.9	25.7	4.6	86.5	138.0	109.0	3.4	Normal
5*	1	0	110	21.0	15.0	40.1	4.8	71.5	144.5	117.0	5.1	Normal
	8	150	74	62.5	17.4	40.5	6.2	70.2	126.5	106.6	8.0	Symptoms
	9	100	No samples. Eats full ration.									Active
	10	50	Appears and acts normal.									
	11	0	106	11.6	10.5	29.4	5.0	75.0	148.0	113.0	3.5	Normal

* Intramuscular injections.

electrolyte determinations were not made the first twenty-four hours, except in Dog 3. At this time, the serum Na had not risen to its initial value although the blood urea nitrogen and serum K had declined to normal and hemodilution was evident. The animal was symptom-free and quite active. Dog 5 differed from the other members of the series in that he was restored to normal by intramuscular injections of 2-methyl FF representing 300 μg. administered over sixty hours. All symptoms had disappeared within six hours after the first injection.

DISCUSSION

It is clear that substitution of fluorine for hydrogen at C-9 greatly increases the Na retaining capacity of 2-methyl FF over that of methyl F. Assays previously conducted in this laboratory (5–7) showed that the following compounds were the most active corticoids for maintaining the life of adrenalectomized dogs: aldosterone, fluorocorticosterone acetate and 1-dehydro-9α-fluorohydrocortisone. The MMD for these steroids ranged from 6.2–12.5 μg./dog/day. In view of the data presented in Table 1, it is evident that 2-methyl FF is superior in potency to any other of the naturally occurring or synthetic derivatives of adrenal cortical hormones. A similar conclusion has been reached by others (2–4), from studies on rats, dogs and human patients. This modification of hydrocortisone is at least 4 times more potent than aldosterone, since the MMD of this latter steroid is 6–12 μg./dog/day, whereas but 1.5 μg./day of 2-methyl FF is required to maintain health in the adrenalectomized dog. Moreover, this

compound is 166 times more active than DOC, since approximately 250 μg./day of this latter steroid is necessary for maintenance.

It seems probable that 2-methyl FF derives some of its extreme effectiveness from the fact that it is a long acting drug. Liddle and Richards (2–3) called attention to this property of the steroid and suggested that it might be due to the slow rate at which 2-methyl steroids are metabolized to inactive forms.

The miniscule daily requirement of the dog lacking adrenals of 2-methyl FF, although surprising, is not more so than the small amount of this corticoid needed for revival of the animal from severe insufficiency. The efficacy of the compound in this respect is not equalled by that of any known adrenal steroid. It requires but a few hours following i.v. injection for complete restoration of normal activity in animals in which death was imminent. The potency is such that an i.v. dose of 250 μg. is sufficient to effect profound changes in arterial pressure, serum Na, Cl and K. One of the writers (W. W. S.) reported striking and perhaps equally dramatic results by using the water soluble glucoside of DOC to revive dogs from the prostration of adrenal insufficiency (8). However, in these earlier experiments massive doses (500 mg. or more) of the glucoside were administered over several days. Such a dose is 2000 times greater than is necessary if 2-methyl FF is used.

SUMMARY

Two analogs of hydrocortisone: 2-methylhydrocortisone and 2-methyl-9α-fluorohydrocortisone were assayed on long term adrenalectomized dogs. The minimum maintenance dose of 2-methylhydrocortisone was 500 μg./dog/day which is twice that of desoxycorticosterone; hence this derivative of hydrocortisone exhibited less mineralocorticoid potency in these tests than desoxycorticosterone. 2-methyl-9α-fluorohydrocortisone, on the other hand, is the most powerful steroid known for life maintenance and Na-retention. The minimum maintenance dose for the 3 animals studied was 1.5 μg./day which is 166 times less than the daily requirement for desoxycorticosterone and is at least 4 times less than that of aldosterone. 2-methyl-9α-fluorohydrocortisone is extremely effective in reviving dogs from severe insufficiency and restoring them to normal activity in record time. A dose of 150 μg. i.v. is adequate to cause disappearance of symptoms within a few hours, but an additional 100 μg. is necessary to restore to normal the distorted serum electrolyte pattern. Because of its high potency and prolonged action this analog of hydrocortisone should be a valuable tool for study of adrenal problems.

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THE ACTH-RELEASING ACTIVITY OF EXTRACTS OF THE POSTERIOR LOBE OF THE PITUITARY *IN VIVO*[1]

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A GREAT deal of evidence indicates that the release of pituitary adrenocorticotrophin (ACTH) resulting from stress is under neural control. In particular, it appears likely that the hypothalamus constitutes a major pathway for excitation of ACTH release (1). This conclusion has been supported by experiments in which portions of the hypothalamus have either been stimulated or destroyed, resulting in an increase or decrease of ACTH release, respectively (2–7).

The precise pathway by which the hypothalamus triggers ACTH release has been the subject of active investigation. A direct secreto-motor innervation of the adenohypophysis appears to be unlikely since the adenohypophysis is possessed of only a scanty innervation at best (8), and section of any direct neural pathway from the hypothalamus fails to block ACTH secretion (9, 10). This leaves a neuro-humoral pathway as the most likely mechanism for nervous control.

It has been proposed by a number of workers that the release of ACTH is under the control of the antidiuretic hormone (ADH) liberated from the neurohypophysis (11, 12, 13, 14). The following evidence supports this view: i. Release of ADH occurs during the stressful situations associated with release of ACTH (13, 15, 16), and this release of ADH probably occurs quickly enough to account for the rapidity of release of ACTH (13, 17, 18). ii. There is a correlation between the reduction in ADH release, as judged by severity of diabetes insipidus, and the reduction in ACTH release in rats with hypothalamic lesions (6, 14). iii. Pitressin, a commercial extract containing the pressor-antidiuretic activity of the posterior lobe, is effective in inducing ACTH release in rats with hypothalamic lesions which block the response to non-specific stimuli (14). The dose of Pitressin required to elicit ACTH discharge in rats with lesions is very large, suggesting that the amount of endogenously liberated ADH would be effective only if the ADH reached the pituitary in high concentration via the hypophysial portal veins after its secretion in the median eminence of the tuber cinereum.

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Recently, the ACTH-releasing action of Pitressin has been confirmed in experiments where Pitressin has been added directly to the incubation media of pituitaries *in vitro* (19, 20, 21), but here the activity of Pitressin has been ascribed to the presence of a contaminant in the extracts (19, 20, 21).

It seemed of interest to reinvestigate the effect of Pitressin on ACTH release *in vivo*, using animals in which the ACTH discharge from noxious stimuli had been blocked by pharmacological means as well as by hypothalamic lesions, to see whether the ACTH-releasing effect of Pitressin could be demonstrated under all circumstances. In addition, experiments were designed to determine if this ACTH-releasing activity of posterior lobe extracts *in vivo* could be separated from the pressor-antidiuretic activity of the extracts.

METHODS

Male rats of the Wistar strain, weighing between 270–330 gm., were used throughout these experiments. The ACTH-releasing effect of a given procedure was evaluated by means of adrenal ascorbic acid depletion (22, 4). Left adrenalectomy was performed first in all experiments to obtain a control level of adrenal ascorbic acid. All drugs were administered immediately after left adrenalectomy. The right adrenal was removed one hour after unilateral adrenalectomy or injection of the test drug, and the adrenal ascorbic acid depletion was taken as an index of the ACTH-releasing effect of the procedure.

Two methods of pharmacological blockade of ACTH release were used: 1) Nembutal-morphine anesthesia (23) or 2) hydrocortisone acetate overdosage (24). In the first instance, rats were anesthetized with Nembutal (4 mg./100 gm., intraperitoneally (i.p.)) and 10 minutes later were injected with morphine sulphate (2 mg./100 gm., i.p.). Ten minutes after injection of the latter, unilateral adrenalectomy was performed. With hydrocortisone overdosage, hydrocortisone acetate was administered i.p. at a dose of 8 mg./100 gm. rat. Four hours later experiments were begun. In all experiments except those with Nembutal-morphine anesthesia, the procedures were performed under ether anesthesia. All normal animals were handled for 3 or more days prior to experiment to decrease the ACTH discharge from the injection of the blocking agents.

Bilateral hypothalamic lesions were produced as previously described with the Krieg stereotaxic instrument (4, 14). Lesions were produced 6.2 mm. rostral to the right ear bar, 0.6 mm. from the mid-line and 0.2 mm. from the base of the skull, using a current of 6 milliamperes applied for 17 seconds on each side of the mid-line. An insulated, nichrome wire with a flat, non-insulated tip was employed as the electrode, the indifferent electrode consisting of contact points of the animal with the stereotaxic instrument. Water intake was measured for 2 or more days prior to testing in all rats with lesions. Hypophysectomized rats, obtained from Hormone Assay laboratories, were tested 48–72 hours post-hypophysectomy.

The intravenous (i.v.) injection of one of the following drugs was performed during a period of approximately 3 minutes: ACTH (Armour), 5 mU.; commercial epinephrine, 5 μg.; histamine acid phosphate, 3 mg.; Substance P, 2.5 mg.; and Pitressin, Pitocin and Protopituitrin in varying doses.[2] Epinephrine and ACTH were also administered

[2] Substance P, Lot #2678-X25, Pitressin, Lot #168941, and Protopituitrin, Lot #194582, were very kindly supplied by Dr. D. A. McGinty of Parke, Davis & Co.

i.p. at a dose of 0.05 mg. and 10 U., respectively. Pitressin in oil was injected subcutaneously once daily for 2 weeks in rats with permanent diabetes insipidus.

Pitressin was subjected to ascending chromatography for 18 hours on Whatman #1 filter paper using the system: 2-2′oxy-diethanol, 10 ml.; acetone, 60 ml.; 0.5% aqueous urea, 30 ml., as described by Guillemin and Hearn (20, personal communication). An adjacent strip was developed with 0.25% ninhydrin in acetone by heating at 80° C overnight. Various portions of the chromatogram were eluted 4 times with five ml. portions of either distilled water or 0.25% acetic acid. Recoveries of vasopressor activity by either method were roughly comparable but incomplete. After centrifugation to remove bits of filter paper, and addition of sodium chloride to make the solution isotonic, the eluates were injected i.v. into rats with hypothalamic lesions. Assay of pressor activity was carried out in the dibenamine-treated, anesthetized rat as described by Dekanski (25).

RESULTS

Pharmacological Blockade of ACTH Release.

A. *Nembutal-morphine anesthesia.* Nembutal-morphine anesthesia was found to suppress completely the ACTH release normally induced by unilateral adrenalectomy as judged by adrenal ascorbic acid depletion (Table 1A). This confirms the findings of Briggs and Munson (23). Since ACTH produced a near maximal adrenal ascorbic acid depletion in these animals, equivalent to that found in the hypophysectomized rat (Tables 1A, 3), the site of blockade of the pituitary-adrenal response to stress seen in the Nembutal-morphine anesthetized rat is not exerted at the adrenal level but must be exerted either on the nervous system or, less likely, the pituitary itself.

Pitressin was capable of inducing ACTH release in rats blocked with

TABLE 1. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN RATS WITH PHARMACOLOGICAL BLOCKADE OF ACTH RELEASE

Procedure	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal)	
		Mean ±S.E.*	Individual responses
A. Blockade produced by Nembutal-morphine anesthesia			
1. Unilateral adrenalectomy	6	−9±16	−61, 36, −50, −11, 21, 4
2. Unilateral adrenalectomy plus:			
a. Saline, i.v.[1]	7	8±18	110, −11, −28, −16, 3, −32, 32
b. Pitressin, 5 U., i.v.	5	154±24	165, 196, 158, 189, 63
Pitressin, 0.5 U., i.v.	5	101±10	107, 84, 120, 70, 122
Pitressin, 0.25 U., i.v.	5	65±19	20, 137, 62, 56, 53
c. Epinephrine, 0.05 mg., i.p.[1]	7	93±32	222, −13, 79, 21, 72, 183, 85
d. ACTH, 5 mU., i.v.	5	143±19	178, 197, 109, 100, 129
B. Blockade produced by hydrocortisone acetate overdosage			
1. Unilateral adrenalectomy	10	22±15	39, 41, 84, 18, 9, −38, 0, −1, −36, 108
2. Unilateral adrenalectomy, plus Pitressin, 0.5 U., i.v.	10	80±14	34, 89, 110, 67, 186, 80, 37, 69, 25, 98

*S.E. = Standard error of the mean.
[1] i.v. = intravenously, i.p. = intraperitoneally.

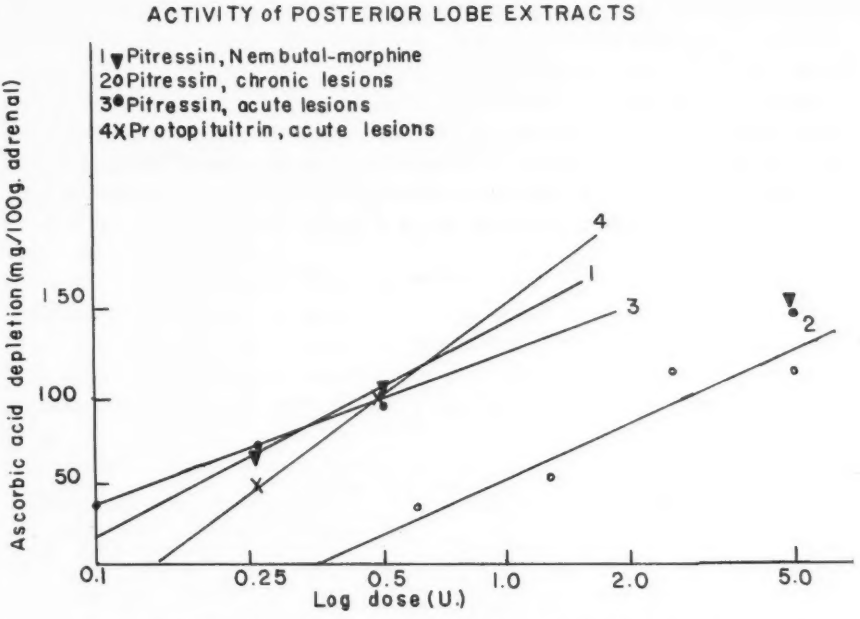

FIG. 1. ACTH-releasing activity of posterior lobe extracts in rats with Nembutal-morphine blockade or with hypothalamic lesions.

Nembutal-morphine, when injected i.v. at a dose of 0.25–0.5 pressor units (U.) (Table 1A), and a linear log dose-response curve was obtained (Fig. 1). Briggs and Munson (23) have reported that Pitressin is ineffective after this blocking procedure, but the dose of Pitressin given in their experiments was roughly half the smallest dose used here. The effect of Pitressin seen in the present experiments is not due to ACTH contamination of the extracts since doses of 5 U. of Pitressin do not cause significant ascorbic acid depletion in the hypophysectomized rat (Table 3).

The ACTH-releasing effect of epinephrine has been reported to be abolished in the Nembutal-morphine treated rat (23). A large dose, however, of epinephrine, i.p., produced ascorbic acid depletion in this preparation (Table 1A).

Nembutal-morphine anesthesia is capable of blocking the ACTH release to severe, non-specific stress as epitomized by unilateral adrenalectomy; however, Pitressin and epinephrine can break through this blockade and produce ACTH release in such animals either by breaking through the blockade imposed by Nembutal-morphine or by a direct action on the adenohypophysis.

B. *Hydrocortisone overdosage.* Rats injected i.p. with 8 mg./100 gm. of hydrocortisone acetate failed to react to unilateral adrenalectomy with a significant ascorbic acid depletion in contrast to the behavior of the normal

rat (Table 1B). An occasional rat may have shown a response in spite of the i.p. injection of the steroid. Intravenous Pitressin at a dose of 0.5 U./rat overcame the blockade and produced an ascorbic acid depletion of 80 ± 14 mg.%, a result significantly different from zero ($P < 0.01$) and from the results with unilateral adrenalectomy alone ($P < 0.02$) (Table 1B). It appears that Pitressin is still capable of eliciting ACTH discharge in the rat with hydrocortisone-induced inhibition of the pituitary-adrenal stress response, a result in agreement with the findings of Porter (24).

Rats with diabetes insipidus as a result of hypothalamic lesions.

A. *Permanent diabetes insipidus (chronic lesions).* It has been reported previously that rats with permanent diabetes insipidus, drinking more than 200 ml. of water per day, do not respond to the stress of unilateral adrenalectomy under ether (14). Table 2A summarizes the results which

TABLE 2. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY VARIOUS STIMULI IN RATS WITH HYPOTHALAMIC LESIONS

Procedure	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal)	
		Mean ±S.E.*	Individual responses
A. Rats with severe permanent diabetes insipidus (chronic lesions)			
1. Unilateral adrenalectomy	56	−14 ± 5	
2. Unilateral adrenalectomy plus:			
a. Histamine acid PO^4, 3 mg., i.v.[1]	6	30 ± 17	−2, 24, 59, −3, 99, 6
b. Epinephrine, 5 μg., i.v.	4	1 ± 10	28, 3, −12, −16
c. Pitocin 5U., i.v.	5	−4 ± 10	−15, −24, 16, 9, −16
1 U., i.v.	4	2 ± 12	−17, 30, 0, 22
d. Pitressin in oil, s.c.[1]	6	7 ± 9	15, −23, 33, −4, 28, −5
e. Aqueous Pitressin			
5 U., i.v.	12	112 ± 18	91, 50, 244, 179, 177, 178, 151, 101, 103, −22, 34, 77
2.5 U., i.v.	3	110	89, 216, 25
1.25 U., i.v.	3	52	63, 52, 42
0.61 U., i.v.	4	34 ± 19	79, −16, 39, 34
f. ACTH			
10 U., i.p.	5	158 ± 28	154, 183, 235, 92, 124
5 mU., i.v.	9	97 ± 28	202, 58, 103, 45, −45, 69, 134, 159, 147
B. Rats with the transient phase of diabetes insipidus (acute lesions)			
1. Unilateral adrenalectomy	6	−6 ± 8	−2, −4, 12, 10, −42, −10
2. Unilateral adrenalectomy plus:			
a. Histamine acid PO_4, 3 mg., i.v.	7	9 ± 21	−29, −30, 80, −65, 75, 10, 26
b. Epinephrine, 0.05 mg., i.p.	6	−4 ± 12	−26, −4, 47, −21, −30, 9
c. Substance P, 2.5 mg., i.v.	5	8 ± 28	102, −68, 25, −19, 2
d. Aqueous Pitressin			
5 U., i.v.	3	158	116, 199, 160
0.5 U., i.v.	6	96 ± 19	108, 141, 147, 18, 80, 80
0.25 U., i.v.	5	74 ± 14	104, 31, 86, 49, 98
0.1 U., i.v.	4	36 ± 19	13, 15, 23, 94
e. Protopituitrin			
0.5 U., i.v.	5	102 ± 17	92, 54, 147, 87, 132
0.25 U., i.v.	5	46 ± 25	145, 32, 21, 6, 31
f. ACTH, 5 mU., i.v.	7	121 ± 19	57, 49, 138, 123, 180, 139, 159

* S.E. = standard error of the mean.

[1] i.v. = intravenously; i.p. = intraperitoneally; s.c. = subcutaneously.

have been obtained with various stimuli in these animals. A large dose of ACTH gave a uniform ascorbic acid depletion in these rats (Table 2A, 2)f). The smaller dose of 5 mU. of ACTH, which produced a near maximal response in hypophysectomized rats and rats with blockade due to Nembutal-morphine, produced a more variable ascorbic acid depletion in these rats with permanent diabetes insipidus. The mean response was 97 ± 28 mg.%, a value which was significantly different from zero and was not significantly less than that found in the hypophysectomized rats. The failure of rats with chronic lesions to respond to stress, therefore, can not be ascribed to adrenal insensitivity to ACTH, though this may be a factor in some of these rats. Pitressin in oil in a dosage sufficient to control the diabetes insipidus of these rats over a two week period did not alter the ex-

TABLE 3. ADRENAL ASCORBIC ACID DEPLETION PRODUCED BY PITRESSIN AND ACTH IN HYPOPHYSECTOMIZED RATS

Procedure	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal)	
		Mean ±S.E.*	Individual responses
1. Unilateral adrenalectomy plus:			
a. Aqueous Pitressin, 5 U., i.v.	11	9 ± 9	−8, 10, 61, 40, 9, 22, 17, −33, −24, 35, −26
b. ACTH, 5 mU., i.v.	5	123 ± 16	163, 100, 151, 79, 120

* S.E. = Standard error of the mean.

pected result of unilateral adrenalectomy in the animals with lesions (Table 2A). Thus, diabetes insipidus, per se, is not responsible for the blockade in ACTH release from stress seen in these rats.

Pitressin induced ACTH release in the rat with chronic lesions, but the dose required to elicit this effect appeared to be of the order of 1–5 U., i.e., much larger than that required in rats with pharmacological blockade of ACTH release (Table 2A). The log dose-response curve obtained with varying doses of Pitressin was shifted to the right but was of similar slope on comparison with the curve obtained in the Nembutal-morphine treated rat (Fig. 1). Pitocin, a commercial preparation of oxytocin, lacked ACTH-releasing activity as did epinephrine and histamine under the conditions of these experiments (Table 2A).

B. *Rats with transient diabetes insipidus (acute lesions).* Because of the rather erratic responses to Pitressin and ACTH found in the rats with permanent diabetes insipidus, it seemed of interest to evaluate the release of ACTH in rats operated on only 48 hrs. previously and exhibiting the transient phase of diabetes insipidus. During the 48 hour postoperative period the animals usually drank from 200–1000 ml. of H_2O. Animals with water intakes in excess of 110 ml. in the first 24 hours post lesions were used. The results with the acutely lesioned rat are shown in Table 2 B. Uni-

lateral adrenalectomy produced no ascorbic acid depletion whatsoever in these rats (Table 2B). In contrast, four rats with lesions of the hypothalamus designed to spare the supraotico-hypophysial tract gave an ascorbic acid depletion of 132 ± 18 mg.%, a response significantly different from zero. It appears that the failure of ascorbic acid depletion in rats with acute lesions is a consequence of the specific, hypothalamic lesions present in these rats.

In the rat with acute lesions, ACTH elicited a good response, which was not significantly less than that found in hypophysectomized rats. Therefore, the site of the blockade in ascorbic acid depletion in these rats with acute lesions is not at the adrenal cortex. Pitressin gave a reproducible ascorbic acid depletion in these rats, with a minimal effective dose of roughly 0.25 U./rat. Results obtained with varying doses of Pitressin yielded a linear log dose-response curve which was similar to that obtained in the Nembutal-morphine anesthetized rat (Fig. 1). The sensitivity of the rat with acute lesions to Pitressin is, therefore, similar to that found in the Nembutal-morphine anesthetized rat and the hydrocortisone-inhibited rat. Epinephrine was ineffective in these animals even when given i.p. in the dose which elicited ACTH release in the Nembutal-morphine anesthetized rat. Histamine was without effect, as was Substance P of von Euler and Gaddum (Table 2B).

It is apparent that the rat with acute lesions possesses several advantages for testing the ACTH-releasing activity of possible neuro-humors over the rat with chronic lesions. Tests with the acute animal are much less time consuming because of the short postoperative period. The responses obtained appear also to be somewhat less variable and, lastly, the rat with the acute lesions fails to respond to a variety of nonspecific stimuli while maintaining a normal response to ACTH. Therefore, the ACTH release from Pitressin may represent the response to a specific neuro-humoral agent in these test animals.

Attempts to separate ACTH-releasing activity from pressor activity. It appeared of interest to determine whether or not the ACTH-releasing activity of posterior lobe extracts could be separated from their pressor activity as reported in the use of *in vitro* techniques of measuring ACTH release (19, 20, 21). Rats with acute lesions were used to assess ACTH-releasing potency of the extracts. Protopituitrin, a less purified extract of posterior lobe activity possessing equal pressor and oxytocic unitage (15 pressor and oxytocic U./mg.), was evaluated first. Assay of this material at 2 dose levels in rats with acute lesions indicated that the potency of Protopituitrin in ACTH release is not significantly different from that of Pitressin if the administered dose of both substances is expressed in pressor units. In other words, the ACTH-releasing activity of Protopituitrin is accounted for by its content of pressor activity.

Ascending chromatography of Pitressin was carried out using the system

proposed by Guillemin and Hearn (20) to test further the possibility that pressor and ACTH-releasing activity could be separated. The results are shown in Table 4. With this method, most of the ninhydrin positive material was concentrated with an Rf near unity. Lesser amounts of ninhydrin positive material were found with Rf's varying from 0.4–1.0. The paper was arbitrarily cut into 4 portions, eluted, and tested in rats with acute lesions of the hypothalamus. The eluates were also tested for pressor activity in the dibenamine-inhibited, anesthetized rat (25). As can be seen in Table 4, ACTH-releasing activity was found in eluates from that portion of the chromatogram with Rf 0.3–0.8. These eluates also produced con-

TABLE 4. ACTH RELEASING ACTIVITY OF VARIOUS FRACTIONS OF THE CHROMATOGRAM OF PITRESSIN USING RATS WITH ACUTE LESIONS

Rf value	Dosage arbitary units)	No. of rats	Adrenal ascorbic acid depletion (mg./100 gm. adrenal)		P† Value	Pressor response
			Mean ±S.E.*	Individual responses		
0.0–0.3	1	6	−32 ±27	−34, −87, −122, 62, −22, 9	—	0
0.3–0.5	1	4	65 ±19	10, 79, 76, 94	<0.01	
	1	6	60 ±12	114, 63, 62, 27, 45, 50	<0.01	++
0.5–0.8	1	5	76 ±35	153, 61, −39, 144, 80	<0.05	
	1	5	60 ±24	−14, 86, 12, 134, 84	<0.02	++
0.8–1.0	1	3	10	−6, 12, 26	—	
	2.5	4	− 4 ±22	−67, 39, −3, 13	—	0
	5.0	6	10 ±11	−6, 6, 42, −20, −9, 44	—	
0.9 (fraction D)[1]	0.5 mg.	7	46 ±15	36, 94, 38, 11, 106, 31, 10	<0.02	
	1.0 mg.	6	18 ± 9	−15, 44, 37, 15, 1, 25	<0.1	

* S.E. =standard error of the mean.

† P value computed using "Student's" t test, comparing the mean response obtained here with that found when the rats were unilaterally adrenalectomized.

[1] Prepared and sent to us by Dr. Guillemin. This material was active on assay, *in vitro*

striction of peripheral vessels as indicated by blanching of the skin (of feet and ears), and an elevation of the blood pressure when tested in the dibenamine-inhibited, anesthetized rat. Eluates from that portion of the chromatogram with Rf 0.0–0.3 were ineffective both in eliciting ACTH discharge and in raising blood pressure. The fraction with Rf 0.8–1.0 was also ineffective in eliciting ACTH discharge, even when injected at a dose 5 times that used for the other eluates, and appeared to have virtually no pressor activity. In other words, both ACTH-releasing activity and pressor activity were associated with the same portion of the chromatogram with an Rf 0.3–0.8.

The results reported with *in vivo* assay in rats with acute lesions are at variance with the findings of Guillemin and Hearn obtained with pituitaries *in vitro* (20). As reported here, they found ACTH-releasing activity from the portion of the chromatogram containing pressor activity, although they found the major activity to be associated with the fraction with Rf 0.95. But in the present experiments this latter fraction was inactive *in*

vivo, even at a dose level 5 times that sufficient to evoke activity from the two fractions of the chromatogram with pressor activity. Thus, *in vivo* results, in contradistinction to those results reported *in vitro*, fail to give any evidence of separation of pressor activity from ACTH-releasing activity.

In hopes of clarifying this discrepancy, Dr. Guillemin very kindly supplied me with some of his fraction D, prepared from that portion of the chromatogram with Rf near unity and active on assay *in vitro*. This has been evaluated at 2 dose levels in rats with hypothalamic lesions (Table 4). Doses of 0.5 and 1.0 mg. gave ascorbic acid depletions of 46 ± 15 and 18 ± 9, respectively. The first result is significantly different from zero but of an order of magnitude such that it must be considered a doubtful response. The result with the higher dose is not significantly different from zero. Similar responses are obtained with 0.002 mg. (0.1 U.) of Pitressin and 0.016 mg. (0.25 U.) of Protopituitrin. The sensitivity of rats with acute lesions to Pitressin and Protopituitrin appears to be roughly 200–400 and 30–60 times greater than that to fraction D of Guillemin and Hearn.

Miscellaneous observations.

A. *Changes in body weight after hypothalamic lesions.* Rats with acute lesions either gained or lost small amounts of weight during the 48 hour postoperative period; the mean weight loss was 3.5 gm. Weight changes in rats with chronic lesions were similar to those reported earlier, nearly all animals gaining weight during the postoperative period.

B. *Changes in adrenal weight after hypothalamic lesions.* Adrenal weights of rats with acute lesions were 17% larger than the glands from control animals used for the experiments with Nembutal-morphine and hydrocortisone. This weight increase appears to represent an adrenal cortical response of the rat with lesions to the combined stress of operation and high water turnover, which can occur even in the presence of hypothalamic lesions capable of blocking the acute response to stress. This observation is reminiscent of the increase in adrenal weight found in rats with very severe permanent diabetes insipidus and of the attenuated increase in adrenal weight seen after unilateral adrenalectomy in rats with permanent diabetes insipidus (6, 14, 26).

C. *Location of hypothalamic lesions.* Serial sections through the hypothalamus and pituitary of four of the rats with acute lesions revealed that the lesions lay in the median eminence in each case. Except for a small infarct estimated to occupy 10% of the volume of the adenohypophysis in one rat, the pituitaries showed no definite abnormalities after staining with hematoxylin and eosin.

D. *Resting values for adrenal ascorbic acid concentration.* The mean initial ascorbic acid concentration of rats with Nembutal-morphine anesthesia was 404 mg.%, while the value for rats treated with hydrocortisone was 403 mg.%. Elevated values of 525 and 472 mg.% were found in rats with acute and chronic lesions, respectively.

DISCUSSION

Several methods of inhibiting the pituitary-adrenal response to stress are now known. Anesthetic agents, such as the combination of Nembutal-morphine anesthesia, were first shown to block ACTH release by Briggs and Munson (23). This blocking action has been confirmed in the present study and that of Ohler and Sevy (27). Tranquilizing drugs are also effective in blocking ACTH release (28). Large doses of cortical steroids were first shown to have an inhibitory effect on ACTH release from acute stimuli by Sayers and Sayers (29), and more recently Hodges (30) and Porter (24) have shown that desoxycorticosterone and hydrocortisone, respectively, can block the response to the severe stimulus of unilateral adrenalectomy. Our results with hydrocortisone are in agreement with those of Porter. Lastly, hypothalamic lesions can block the acute discharge of ACTH in response to severe acute stress. Results indicating only an incomplete blockade from hypothalamic lesions (31) may be explained on the assumption that the lesions were incomplete.

The ideal assay animal to test for possible neuro-humoral activators of ACTH release would be one in which the ubiquitous pituitary-adrenal response to stress was completely blocked while leaving unimpaired the sensitivity of the pituitary and adrenal to the neuro-humor and ACTH, respectively. In the present study examples of several means of inhibiting the stress response have been used. The Nembutal-morphine anesthetized rat appears to be unsatisfactory for assay of neuro-humors since it responds both to epinephrine and to Pitressin. This suggests that any severe stimulus can break through the blockade imposed by Nembutal-morphine anesthesia. Although the adrenals of these animals are sensitive to ACTH, it is, nevertheless, impossible to determine the precise site of action of this blockade, though it is presumably on the central nervous system, rather than the adenohypophysis.

The hydrocortisone-inhibited rat has not yet been fully evaluated. Its sensitivity to ACTH has not been tested (the sensitivity of the desoxycorticosterone-inhibited rat appears to be about normal (30)), and other stressful stimuli have not been evaluated. Even if this rat should prove unresponsive to all nonspecific stimuli, it would be difficult to determine the site of inhibition directly. If it is granted that vasopressin (ADH) is the specific neuro-humor, then the effectiveness of Pitressin in spite of this blockade argues for a site of hydrocortisone inhibition proximal to the pituitary, at least in acute experiments. However, data obtained in experiments where changes in cortical steroid concentration are maintained over a protracted period of time in both rats and dogs suggest that a part of the inhibitory effect of these steroids may be exerted on the hypophysis itself (26, 32).

The rat with acute lesions offers many advantages over the animal with chronic lesions as an assay animal for possible neuro-humoral agents. It is more convenient to use since the time between operation and testing is only

48 hours. It is unresponsive to nonspecific stimuli, yet retains its sensitivity to ACTH, and there appears to be less variation in its response than in the rat with chronic lesions. The reason for the greater sensitivity of the rat with acute lesions to Pitressin is not apparent.

Indeed, rats with acute lesions may be the most suitable test animal for neuro-humoral agents. The lesion is in the hypothalamus, and this is presumably the main site of blocking action, though some decrease in the sensitivity of the pituitary to the neuro-humor can not be ruled out. In spite of the absence of response to nonspecific stimuli, the sensitivity of the rat with acute lesions to Pitressin is comparatively great and the same as that found in both the Nembutal-morphine and hydrocortisone-inhibited rat. All three types of test animals respond to a dose of Pitressin which is only twice that which is effective in intact rats (23). Therefore, it is reasonable to assume that all three blocking procedures suppress the nonspecific response from this drug, and that the minimal effective dose of Pitressin found here is that required to exert an action directly on the pituitary.

The results of assay of various drugs in rats with either acute or chronic lesions leads to rather definite conclusions as to the nature of the hypothalamic neuro-humor responsible for ACTH release. It appears unlikely that epinephrine, histamine, Substance P or oxytocin is active in this regard. Results obtained with Pitressin, Protopituitrin, and fractions of the chromatogram of Pitressin indicate a parellelism between vasopressor and ACTH-releasing activity in these experiments. The results obtained *in vivo* favor the concept that the ACTH releasing neuro-humor is vasopressin (ADH), itself, and offer no support for the view that the action of vasopressin in ACTH release is due to a contaminant in the Pitressin (19, 20, 21).

The results obtained *in vivo* raise a question concerning the validity of *in vitro* methods of assay of ACTH-releasing substances. In all of these techniques the substance in question is added directly to the incubating medium, and stimulation is judged only by an increase in the ACTH activity of this medium. Since the pituitary contains large amounts of stored ACTH, a number of foreign agents might be expected to liberate some of the stored ACTH, by altering the permeability of cellular membranes, thus yielding a false positive test. It is possible that some of the discrepancies between *in vivo* and *in vitro* results may be explained in this way.[3]

The results in the present study can be interpreted to mean that ADH is the neuro-humor responsible for stimulating ACTH release *in vivo*. This

[3] In this connection, a mimeographed note sent with a reprint of the paper by Swingle et al. (21) reads, in part, as follows: "The authors wish to state that further investigation leads us to believe the (*in vitro*) test for ACTH releasing substances employed in this paper is: 1) Not specific since other materials besides those discussed may give positive reactions. 2) The test can not be relied upon to invariably give reproducible data."

is consistent with previous evidence indicating a release of ADH in conditions where ACTH release occurs (13, 15, 16), and with the defect in ACTH release occurring in the ADH deficient rat with diabetes insipidus (6, 14). The final proof of the hypothesis that ADH releases ACTH under physiological conditions will depend on a demonstration that amounts sufficient to activate the gland directly actually reach the pituitary either by the hypophysial portal vessels or the general circulation.

SUMMARY

Nembutal-morphine anesthesia, administration of large doses of hydrocortisone acetate, or production of hypothalamic lesions blocks the pituitary-adrenal response to acute stress as judged by adrenal ascorbic acid depletion.

The Nembutal-morphine treated rat and the rat with hypothalamic lesions are still capable of responding to ACTH with a depletion of ascorbic acid.

Pitressin is capable of evoking ACTH release in rats with blockade of stress-induced ACTH discharge produced by any of these three methods.

Histamine, epinephrine, Substance P, and Pitocin were all ineffective in eliciting ACTH release in the dosage used in rats with hypothalamic lesions.

In rats with acute lesions, tests using Pitressin, Protopituitrin, or chromatographic fractions of Pitressin revealed a correlation between ACTH-releasing and pressor activity.

The rat with acute hypothalamic lesions is proposed as an assay animal for assessing possible neuro-humoral agents.

The evidence obtained *in vivo* supports the hypothesis that ADH may be the neuro-humor responsible for eliciting ACTH discharge.

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NOTES AND COMMENTS

REVERSAL OF ADRENAL REGENERATION HYPERTENSION BY AMPHENONE

Amphenone (1,2-bis (*p*-amino phenyl)-2-methyl propanone-1) has been shown to cause adrenomegaly in the rat in the presence, but not in the absence, of an intact hypophysis (1). Several studies have indicated that this increase in adrenal size may be due to response by the anterior pituitary to a nonfunctional adrenal gland, and low levels of circulating glucocorticoids (2, 3). On the premise that Amphenone caused adreno-cortical depression, it became of interest to know whether this compound would affect the rise in blood pressure, which has been described as a concomitant of adrenal regeneration in the rat.

METHOD

The blood pressure of normal male hooded rats weighing between 80 and 90 grams was determined by a foot cuff method without anaesthesia (4). In an Analysis of Variance applied to this method we have found an Index of Precision of 96% (to be published). Two determinations of the blood pressure at intervals of 3 days served as controls. The animals were then operated on by the method of Skelton (5), i.e. unilateral nephrectomy, right unilateral adrenalectomy and left unilateral adrenal enucleation, under pentobarbital sodium anaesthesia. After recovery the animals were given 1.0% NaCl in tap water to drink, and Purina Fox chow was supplied as the only source of food. The systolic blood pressure of these animals was measured twice each week, and the mean values of these readings for each group of animals with the 95% fiducial limits are given in Table 1. At the times noted therein Amphenone B in aqueous suspension was given once daily by oral gavage at a dose of 200 mg./kg. to the animals of Groups II, III and IV. The blood pressure of these animals was measured in a similar manner.

RESULTS AND DISCUSSION

The results of this study are summarized in the table. It may be seen from the results in Group II that the oral administration of Amphenone after the establishment of hypertension caused a gradual but definite lowering of the blood pressure. When the Amphenone treatment was initiated earlier, and only moderate increases in blood pressure were present (Group III), reversal of the blood pressure to normotensive levels occurred much more promptly. The rats in Group IV, which were treated at 14 days after operation, and before any significant change in blood pressure occurred, did not show the expected rise. It will be noted in all the treated groups that the administration of Amphenone for periods of 4 and 5 weeks did not lower the blood pressure below normotensive levels. Any attempts to explain these results on the basis of the data now in hand must be purely speculative. However, several possibilities are worthy of consideration. It has been reported by Grollman and Harrison (6) that withdrawal of sodium chloride during the development of renal hypertension could prevent further rise in the blood pressure. The fluid intake of these animals has not been measured, however no obvious decrease in intake was noted, and it is thought to be doubtful that a decreased intake of saline could have been maintained by the animals over the extended periods of Amphenone treatment. Amphenone may also be acting via a central mechanism to lower the blood pressure, similar to the effect of Rauwolfia alkaloids. Some credence is lent to this supposition by the demonstration of central depressant activity of Amphenone in the dog and rabbit (7), and the finding of lowered blood pressure in hypophysectomized dogs re-

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TABLE 1. THE EFFECT OF AMPHENONE ON THE BLOOD PRESSURE OF THE RAT DURING ADRENAL REGENERATION

Average systolic blood pressure

Groups	Pre-oper.	Weeks 1	2	3	4	5	6	7	8	9
Group I (18 animals) Controls	107 ±3*	115 ±8	133 ±9	146 ±7	158 ±4	160 ±9	161 ±11	161 ±9		
Group II (8 animals) Amphenone	112 ±4	117 ±6	138 ±7	145 ±8	155 ±10	162 ±12	170 ±10	152 ±8	138 ±7	130 ±10
Group III (7 animals) Amphenone	114 ±4	121 ±8	141 ±6	153 ±8	129 ±9	123 ±10	119 ±8	122 ±9	121 ±8	
Group IV (9 animals) Amphenone	115 ±3	123 ±11	138 ±8	130 ±7	129 ±6	123 ±9	118 ±9	121 ±8		
	Pre-operative	Post-operative		Amphenone treatment						

* 95% fiducial limits.

ceiving intravenous Amphenone (1). However, if such a mechanism plays an important role, the failure of the blood pressure of these animals to fall below normotensive levels is difficult to explain. Adrenal regeneration hypertension has been attributed to an overproduction or imbalance of adrenal cortical hormones by the regenerating tissue. However up to the present time there is no direct evidence to support this concept. The demonstration of depression of adrenal function by Amphenone in the rat and dog (1, 2), suggests that such a mechanism may play a part in explaining our results. Experiments are in progress designed to throw some light on the specific action of Amphenone on adrenal function of hypertensive rats, during the process of adrenal regeneration. This work will be reported elsewhere in detail.

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PLASMA ANTIDIURETIC ACTIVITY OF MARSUPIALS DURING EXPOSURE TO HEAT

Recent investigations have shown that, during acute exposure to heat, an increase occurs in the antidiuretic activity of the plasma of several species of placental mammals (1, 2). Reduced urine flow accompanies this rise in plasma antidiuretic substance (ADS). There is suggestive evidence in the literature that the antidiuretic activity may be due to posterior pituitary antidiuretic hormone (ADH). The pituitary of the kangaroo rat, a small desert rodent, contains more ADH (0.9 mU./μg.) than that of laboratory rats (0.3 mU./μg.) and the concentration of ADH in the urine (up to 50 mU./cc.) is much higher than in the rat and dog, which normally secrete only traces (3). Acute heat stress has been shown to evoke a discharge of ADH from the posterior pituitary of the rat (4).

Animals which possess such a water-saving mechanism employ evaporative cooling for maintaining body temperature in hot atmospheres without endangering cellular hydration. It thus was of interest to determine whether the more primitive mammals—the marsupials—have a mechanism similar to that of the eutheria, for conserving body-water. It has been observed that marsupials have a lower heat tolerance than placental mammals and also make less use of evaporative cooling during heat stress (5, 6, 7, 8).

METHODS

Three species of Australian marsupials were exposed in a psychrometric room to a hot-dry atmosphere for a period of 1–4 hours. Drinking water was allowed until 3 hours before observations commenced. Measurements of plasma ADS and protein were made at ambient temperatures of 20–25° C (control readings) immediately before the animals entered the hot-room and hourly during the period of heat exposure. The species and number of animals observed were:

Bandicoot (*Isoodon obesulus*): 4 animals
Possum (*Trichosurus caninus*): 2 animals
Wallaby (*Setonix brachyurus*): 2 animals with 2 heat exposures on each

Nomenclature is according to Troughton (9).

Antidiuretic activity of plasma was determined by a method, described previously (1), in which intravenous injections are made into an assay rat maintained on a constant water load under ethanol anesthesia. The antidiuretic effect of the plasma was compared with that obtained from known amounts of Pitressin injected into the same assay rat. The method is sensitive to injections of 10 μU. Pitressin and can discriminate does of 5 μU. over the range of 10–80 μU. Pitressin. Plasma ADS below 20 μU./cc. could not be measured since the method lost its sensitivity if the volume of injected fluid in the rat assay exceeded 0.5 cc.

The plasma protein concentration of the blood was estimated by an Abbé refractometer. An indwelling plastic tube was tied into the jugular vein of the experimental animal 2 hours before heating to ensure minimum disturbance during extraction of the blood samples, thereby reducing the possibility of an emotional ADH release.

RESULTS AND DISCUSSION

The findings are summarized in Table 1. Plasma ADS increased in all species during short periods of heating when hemoconcentration, as measured by plasma protein levels, occurred. Periods of heat which were insufficient to produce hemoconcentration did not cause a release of antidiuretic substances.

These findings, which are similar to those we have observed in the rat, sheep, and man (1, 2), suggest that marsupials, like the eutherian mammals, possess a mechanism to meet the continual threat to homeostasis from respiratory and body surface evaporation in a hot environment. Whether the plasma ADS results from neurohypophyseal release of ADH has not yet been determined.

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TABLE 1. ANTIDIURETIC ACTIVITY AND PLASMA PROTEIN (BY REFRACTIVE INDEX) OF MARSUPIAL PLASMA FOLLOWING EXPOSURE TO HOT ATMOSPHERES

	Control		Hot-room				
	ADS (μU./cc.)	Plasma protein (gm./100 cc.)	Air temp. D.B./W.B. ° C	Period (hrs.)	ADS (μU./cc.)	Plasma protein (gm./100 cc.)	Rectal temperature rise ° C
Bandicoot	20	6.3	41/33	1	100	6.1	4.7
	<20	5.0	42/31	1½	60	5.4	3.6
	<20	5.2	41/32	2	80	5.4	4.4
	<20	5.6	41/32	2	70	5.8	4.3
Possum	35	5.1	40/33	2	75	5.4	2.8
	<20	5.6	41/32	2	70	5.8	2.9
Wallaby	<20	5.7	40/31	2	20	5.8	1.4
				4	30	—	1.4
	<20	6.4	40/31	2½	<20	6.4	1.4
				4½	100	—	1.2
	<20	6.6	44/36	2	100	7.7	2.6
				3½	170	—	3.6
	<20	5.4	44/36	2	100	6.7	3.3

SUMMARY

An antidiuretic substance is detectable in the blood of marsupials on exposure to heat. This behavioral pattern resembles that of the more highly evolved placental mammals.

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PROGESTATIONAL ACTIVITY OF VARIOUS STEREOISOMERS OF 19-NOR-PROGESTERONE[1,2]

The conversion of strophanthidin into a 19-nor-11-desoxycorticosterone and 19-nor-progesterone respectively was previously reported (1). Both products were amorphous and were therefore considered impure. They were later (cf. 2) tentatively assumed to possess the 14β,17α-configurations. Whereas the amorphous 19-nor-11-desoxy-10ξ,14β 17α-corticosterone did not produce any corticoid activity (1), the amorphous 10ξ,14β,17α-progesterone apparently surpassed the progestational activity of progesterone (3). The observation of pronounced progestational activity with a compound belonging to the progesterone type and having "unnatural" configurations was unexpected, especially since 17α-progesterone (4) and 14β,17α-progesterone (5) are claimed to be devoid of potency. Hence the repetition of the bioassays with well defined crystalline material appeared indicated. The following compounds have now (6) become available in a crystalline form: 19-nor-11-desoxy-10ξ,14β,17α-corticosterone (I), 19-nor-11-desoxy-10ξ,14β,17α-corticosterone acetate (II) and 19-nor-10ξ,14β,17α-progesterone (III). In addition, very small amounts of a by-product were isolated which possibly represents 19-nor-10ξ,14β-progesterone (IV). It is to be noted that I and III are clearly different from the corresponding 19-nor-compounds having normal configurations (7, 8) which are known to be physiologically highly active (9,10).

TABLE 1

Total dose	Progestational proliferation*		
	III	IV	Progesterone
0.5 mg.	+4; +4; +4	+4	+4; +4; +3
0.25 mg.	+4; +4; +4		+3; +3; +3
0.125 mg.	+3; +4; +3; +4; +4	+4	−; −; −
0.0625 mg.	+3; +2; +2; +4; +3; +1; +2; +1	+4	−; −; −
0.0312 mg.	+1; −; −; −; −; −	+3; +3; +2	
0.0156 mg.	−; +1; −	−; −	

* Each number represents the graded progestational response of one rabbit (cf. Ref. 10).

Compound I was found to possess no mineralocorticoid activity (experiments by Dr. John A. Luetscher, Jr., cf. ref. 6), a result which is in agreement with the observations made earlier (1) with the amorphous product.

We wish now to report on the progestational potency of the crystalline compounds III and IV as demonstrated by the Clauberg assay in the rabbit. The assay procedure has been previously described (10).

The results are presented in Table 1. It is obvious that the potency of each of these isomers is comparable to that previously reported for the "normal" 19-nor-progesterone (8, 10). The latter, in turn, has been found to be 4 to 8 times as active as progesterone (10). Because extremely limited amounts of IV have been available for assay, a precise comparison of its potency with that of III is not feasible. However, the data do warrant the estimation of the potency of IV as being of the same order as that of III. These find-

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[2] This paper is dedicated to the memory of Wolfgang Heubner, Dr. med., Dr. med. h.c., Dr. med. vet. h.c., Dr. rer. nat. h.c., Emeritus Professor of Pharmacology at the Free University of Berlin, who died on February 26, 1957, shortly before his 80th birthday (June 18, 1957).

ings clearly show that in compounds of progesterone type (Δ^4-3-oxo-17-methylketones) "unnatural configurations" do not necessarily preclude high progestational effectiveness. Mention should be made in this connection of the moderate progestational activity of 8α-progesterone (11).

SUMMARY

As determined by Clauberg assay, pure crystalline 19-nor-10ξ,14β-progesterone (IV) and 19-nor-10ξ,14β,17α-progesterone (III) both possess very high progestational potency comparable with that of the "normal" 19-nor-progesterone, thus indicating that in compounds of progesterone type "unnatural configurations" do not necessarily preclude high progestational effectiveness.

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